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Biochemistry of Hemolysin Toxin Activation by Fatty Acylation: Characterization of an Internal Protein Acyltransferase

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BIOCHEMISTRY OF HEMOLYSIN TOXIN ACTIVATION BY FATTY ACYLATION: CHARACTERIZATION OF AN INTERNAL PROTEIN ACYLTRANSFERASE

A Dissertation

Presented to

the Faculty of the Department of Biochemistry and Molecular Biology

James H. Quillen College of Medicine

East Tennessee State University

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy in Biomedical Science

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by

Michael Stephen Trent

December 1998

APPROVAL

This is to certify that the Graduate Committee of

MICHAEL STEPHEN TRENT

met on the

29th day of October, 1998

The committee read and examined his dissertation, supervised his defense of it in an oral examination, and decided to recommend that his study be submitted to the Graduate Council in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biomedical Sciences.

Chair, Graduate Committee

Signed on behalf of
the Graduate Council

Dean, School of Graduate Studies

ABSTRACT

BIOCHEMISTRY OF HEMOLYSIN TOXIN ACTIVATION BY FATTY ACYLATION: CHARACTERIZATION OF AN INTERNAL PROTEIN ACYLTRANSFERASE

by

Michael Stephen Trent

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 post-translational fatty acylation of two internal lysine residues. HlyC, *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. HlyC was thought to serve as an internal protein acyltransferase and remained uncharacterized until now.

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 acyltransfer was assayed. Acyl-ACP was the obligate acyl donor. Acyltransfer was catalyzed by HlyC monomer, and an acyl-enzyme intermediate was detected and shown to catalyze the reverse reaction. The reaction mechanism was examined by steady state kinetics, and the nature of inhibitions by reaction products was determined. The kinetic mechanism of the internal protein acylation was compatible with an uni uni iso uni uni ping pong with isomerization of the F form of the enzyme.

Clues to the chemical mechanism for the acyltransferase were elucidated by both chemical modification studies and site directed mutagenesis of the enzyme. Chemical modification experiments ruled out any critical cysteines, serines, and lysine residues, but suggested a role for histidine(s) and tyrosine(s) in acyltransferase function. In order to

examine the function of specific residues and possibly corroborate the chemical findings, site directed mutagenesis studies of the acyltransferase were empolyed. Seventeen residues that were conserved among 13 different RTX toxin acyltransferases were individually mutated, and the respective HlyCs expressed, and characterized. Residues that were critical for acyltransferase function included Gly 11, His 23, Tyr 70, and Gly 85. As with chemical modification data, mutagenesis ruled out any conserved, essential, cysteines or serines critical for HlyC acyltransferase activity.

DEDICATION

This work is dedicated with love to my wife, Paula. She continues to be the most important person in my life, my best friend. Only the wonderful times that we shared together allowed me to continue with my endeavors in science. True happiness is all about balance and she maintains mine.

Also, this is dedicated to my parents, Mike and Gay. My father always gives me a quote from the Bible when he is proud of me, "A wise son maketh a glad father." Well, that goes both ways. My parents' love and encouragement are never failing and this has always been a source of confidence and happiness for me. Thank you so much for the foundation that has allowed me to obtain my dreams.

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Finally, I would like to express my gratitude to several special students and co-workers. To Greg, Don, Jon, Bob, and Denise, you guys were great. Greg, I always wished we could have spent more time together, I was bored when you left. Don, you always made me feel like I was back home. Jon, I will be seeing you soon and look forward to spending time with you again. Thanks to you and Diane for taking care of Paula and I during the transition to Duke. Denise, it was so nice having you around, especially in class. Keep up the good work and who knows I may be seeing you soon.

All of you are very special people and it brings comfort to know that people of your stature and integrity are in the field of research.

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ABREVIATIONS

ACP	Acyl carrier protein
ACPSH	Acyl carrier with a free prosthetic group thiol
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
BCIP	5-bromo-4-chloro-3-indoyl phosphate
BisTris	Bis[2-hydroxylethyl]iminotris[hydroxymethyl]methane
DEPC	Diethylpyrocarbonate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
HEPES	N-[2-hydroxyl]piperazine-N'-[2-ethanesulfonic acid]
HlyA	Hemolysin A toxin
HlyC	Acyl-ACP-proHlyA acyltransferase
HPLC	High performance liquid chromatography
IPTG	Isopropyl β-D-thiogalactopyranoside
LB	Luria broth
MES	2-(N-morpholino)ethanesulfonic acid
NAI	N-Acetylimidazole
NBT	Nitroblue tetrazolium
NEM	N-Ethylmaleimide
PAGE	Polyacrylamide gel electrophoresis
PCMB	*p*-Chloromercuribenzoate
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PG	Phenylglyoxal
ProHlyA	Hemolysin A protoxin
SDS	Sodium dodecylsulfate
TNM	Tetranitromethane
Tris	Tris[hdryoxymethyl]aminomethane

CHAPTER 1

INTRODUCTION

Pathogenic *Escherichia coli* secrete a toxic protein, hemolysin (HlyA), which binds to and lyses mammalian cell membranes and, at lower concentrations, perturbs cell signal transduction and release of inflammatory mediators. Approximately 50% of *E. coli* causing extraintestinal infections in man produce HlyA, thus it is considered an important virulence factor (*1*, *2*). It is a member of a family of homologous cytolytic toxins produced by Gram negative bacteria. These newly discovered toxins are characterized by the presence of numerous glycine rich nine amino acid repeat units and have been labeled the RTX (repeat in toxin) toxins (*3*). In addition to their unique structural feature, the toxins share a Ca^{2+} dependency for cell lysis, a signal-peptide independent secretion mechanism, a common mode of activation, but they vary in target cell specificity. Various RTX toxins include: enterohemorrhagic 0157:H7 *E. coli* hemolysin (EhxA) (*4*), *Bordetella pertussis* bifunctional adenylate cyclase-hemolysin (CyaA), *Pasteurella hemolytica* leukotoxin (LktA), *Actinobacillus actinomycetemcomitans* leukotoxin (AaltA), and *Actinobacillus pleuropneumoniae* hemolysins (ApxIA and ApxIIA) and leukotoxin (ApxIIIA), with *E. coli* HlyA considered the prototype of the group (*5*).

The *E. coli* HlyA toxin arises from the expression of the *hlyCABD* operon located on either the bacterial chromosome or conjugative plasmids (*6*). The *hlyA* gene product is first synthesized as a 110 kDa inactive protoxin (proHlyA) that is post translationally modified by the *hlyC* gene product, HlyC (*7*). The acylation reaction occurs within the cytosol of the bacterium and requires HlyC for the transfer of a fatty-acyl group from

acyl-acyl carrier protein (acyl-ACP) to proHlyA, converting it to HlyA. Acyl-ACP is the obligate acyl donor with acyl-CoAs not serving as substrates in the reaction (*8*). Like acyl-CoA, the acyl group of acyl-ACP is attached to the protein via a thioester bond on the terminal sulfhydryl of a 4'-phosphopantethiene group that is attached to ACP via a phosphodiester bond to serine 36 (*9*). Acyl-ACP is a small (~9 kDa), acidic protein that was first discovered to function in the fatty acid biosynthetic pathways (*9*). Since then, acyl-ACP has been shown to function as an acyl donor in the synthesis of phospholipids (*10*), lipid A component of lipopolysaccharide (LPS) (*11*), membrane-derived oligosaccharides (*12*), and for the synthesis of lipoylated enzymes as lipoyl-ACP (*13*). After acylation, HlyA is secreted from the bacterium by the action of two specific inner membrane proteins, HlyB and HlyD, which are part of the hemolysin operon, and the outer membrane protein, TolC (*14*, *15*). Acylation is not essential for secretion (*7*), but acylation is the single factor that renders the protein toxic.

Although, the exact role of HlyC was not known when the present study began, it was assumed to function as an acyltransferase. The nature of *in vitro* HlyC-dependent modification of proHlyA was shown to be an internal acylation of the ε-amino groups of lysine residues 564 and 690 forming amide bonds (*16*). The *in vivo* acylation pattern of *E. coli* hemolysin was shown to be the same as the *in vitro* activated toxin (*17*), but the nature of the fatty acid *in vivo* remains unknown. The *in vivo* modification of CyaA, the adenylate cyclase toxin from *Bordetella pertussis*, was shown to be palmitoylation of the ε-amino group of a single internal residue, lysine 983, corresponding to lysine 690 of HlyA (*18*). A summary of the HlyC-dependent HlyA activation by fatty acylation is shown in Figure 1.

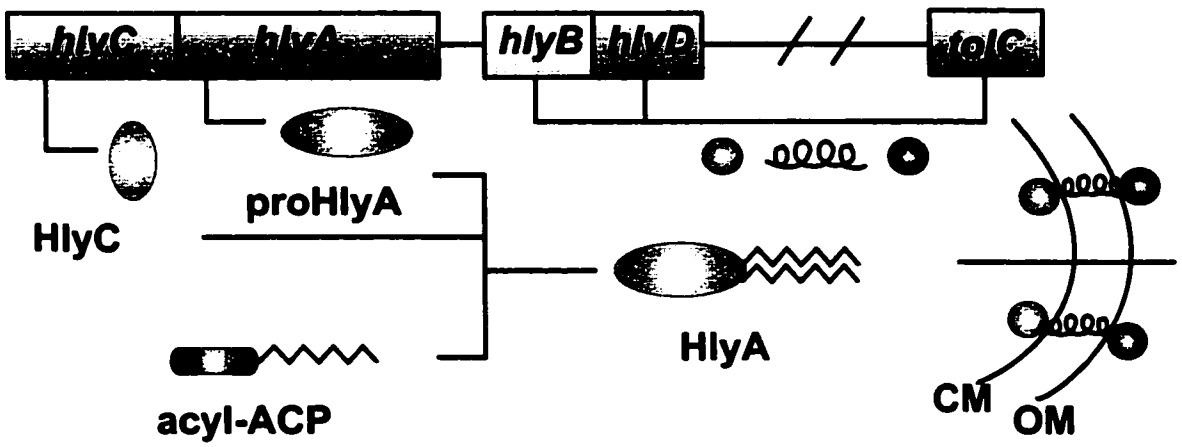


Figure 1: *E. coli* hemolysin (HlyA) acylation and secretion. (CM, cytoplasmic membrane; OM, outer membrane) HlyA is expressed first as the inactive protoxin, proHlyA, which is activated by fatty acylation by the acyltransferase HlyC. Acyl-ACP serves as the acyl donor. HlyA is secreted through a sec-independent pathway by the two gene products of the hemolysin operon, *hlyB* and *hlyD*, and one unrelated protein *tolC*.

Once acylated and in the presence of Ca^{2+}, HlyA has the ability to bind and form cation-specific channels in target cells leading to osmotic lysis (*19*). The nature of cell lysis is not yet fully understood, and the finer points of the subject will not be discussed here. It seems to occur via a two-step mechanism: a reversible membrane adsorption step followed by an irreversible insertion (*20*), the latter requiring a change in HlyA conformation (*21*). It is assumed that membrane insertion is followed by toxin oligomerization within the host cell membrane leading to pore formation (*22*, *23*).

HlyA is a powerful lytic agent, but also of great interest are host cell responses elicited at subhemolytic doses. For example, HlyA induces leukotriene production and phosphoinositide hydrolysis in human neutrophils (*1*), stimulates nitric oxide production in porcine endothelial cells (*24*), increases production of interleukin (IL)1-β and tumor necrosis factor (TNF) in human monocytes (*2*); and induces the rapid and massive shedding of the LPS (CD14) and the IL-6 receptors (*25*). In addition to these responses, HlyA and other RTX toxins have been shown to induce apotosis in human lymphocytes (*26*, *27*).

The various RTX toxins differ in cell and host specificity and are divided into two groups, hemolysins and leukotoxins. For example, HlyA and the hemolysins ApxIA and ApxIIA, have a wide range of specificity including erythrocytes and leukocytes of animals and humans; whereas, the LktA and AaltA leukotoxins have no effect on erythrocytes, but are powerful toxins for a narrow range of phagocytic cells of ruminants and humans, respectively (*28*). The differences in target cell sensitivity for the RTX toxins could be attributed to several reasons including target cell receptor recognition, membrane composition, and membrane repair mechanisms. Recently, Lally and

colleagues showed evidence that the AaltA leukotoxin and HlyA bind to the CD11a-CD18 integrin (lymphocyte function-associated antigen 1) on human cells, possibly functioning as a receptor (*29*).

HlyA toxicity is Ca^{2+} dependent with the RTX toxin nonapeptide repeat unit (Leu-X-Gly-Gly-X-Gly-(Asn/Asp)-Asp-X) functioning as a Ca^{2+}-binding motif via the negatively charged aspartate residues (*30*, *31*). HlyA contains 11-17 repeat units, depending upon how much one adheres to the consensus sequence, between amino acids 739 and 849 (*32*). Ca^{2+} binding is not dependent upon HlyC modification and the necessity of Ca^{2+} for HlyA activity is not fully understood. Although Ca^{2+} binding has not been shown to induce a large conformational change in HlyA, there are associated changes in surface hydrophobicity in the Ca^{2+} bound state (*33*) and Ca^{2+} induced conformational changes have been shown for CyaA (*34*, *35*).

The hemolysin system provides a unique opportunity to study an internal acylation of a protein. Internal fatty acylation of proteins is recognized as a means of modifying protein behavior in biological regulatory mechanisms such as signaling, and many toxins exert their effects through distortion of cellular signaling mechanisms. Numerous instances of eukaryotic protein fatty acylation have been reported (*36*) and two classes of acylproteins are found in the literature. One is typified by 14 carbon myristic acid in an amide-linkage to the N-terminal glycine of the target protein following removal of the initiator methionine. The reaction is catalyzed by the ubiquitous myristoyl-CoA: protein N-myristoyl transferase and has been thoroughly characterized (*37*). The second group contains primarily hexa- or octadecanoic acids in ester linkages

with internal cysteine or threonine residues, and with less frequency in an amide linkage to lysine residues (*38*, *39*).

The acyltransferases catalyzing these internal acylations have been elusive and remain for the most part uncharacterized; although, recently two protein palmitoyl acyltransferases that transfer palmitoyl groups to H-Ras and spectrin have been purified (*40*, *41*). Palmitoylation of proteins at the thiol group of cysteine residues results in thioester bonds which are labile in nature, and a thioesterase that removes palmitate from H-Ras and Gα subunits has been cloned (*42*). Unlike a thiol ester, the amide linkage of acyl groups results in a stable lipid modification. HlyA and other members of the RTX toxin family are the only known prokaryotic proteins that are post-translationally modified by internal amide fatty acylation. There are four eukaryotic proteins that are known to have internal acylated lysine residues: TNF-α (*43*), IL-1α, IL-1β (*44*), and cytochrome *c* oxidase (*45*). Also, two integral membrane proteins, nicotinic acetylcholine receptor and the insulin receptor, are speculated to contain an internal amide linked fatty acid, but the sites of acylation are unkown (*46*, *47*). In time, more internal amide-linked fatty acylations will emerge since metabolic labeling studies predict that only a fraction of acylproteins have been identified (*48*). None of the acyltransferases catalyzing internal amide acylations have been cloned, isolated, or characterized, leaving the characterization of HlyC a unique opportunity.

Comparison of the HlyC sequence to known acyltransferases via a BLAST search revealed no similarities and it has been reported that proHlyA and HlyC are produced in almost equimolar amounts (*49*), confusing the issue that HlyC functions as a catalyst. However, the common scheme of the RTX activation/acylation reaction is demonstrated

by the fact that RTX *C* gene products can activate RTX toxins from different organisms, suggesting that the RTX acyltransferases function in a similar fashion. For example, the hemolysin of *Actinobacillus pleuropneumoniae* (ApxIA), the *Pasteurella hemolytica* leukotoxin (LktA), and the *Bordetella pertussis* adenylate cyclase toxin (CyaA) have all been activated by the *E. coli* HlyC acyltransferase (*50*, *51*, *52*, *53*). An indirect, unstandardized assay of undefined stoichimetry has been used previously to monitor the function of the putative enzyme. Routinely, proHlyA activated by HlyC was incubated with a suspension of erythrocytes in the presence of Ca^{2+}, and the amount of hemoglobin released was monitored. Since the mechanism of hemolysis is unknown, the amount of proHlyA acylated cannot be determined by this method, and the two separate events, acylation and lysis cannot be measured independently.

The role of HlyC in acylation of proHlyA needed clarification. To address the issue of HlyC's putative acyltransferase function, HlyC and proHlyA were subcloned into separate hyperexpression systems and purifed. Using purified, defined acyl-ACP (with radioactive acyl groups) and proHlyA, the acyltransferase activity of HlyC was studied, and the enzyme characterized. The reaction under investigation is shown below.

$$\text{proHlyA} + [^{14}\text{C}]\text{acyl-ACP} \xrightarrow{\text{HlyC}} [^{14}\text{C}]\text{HlyA} + \text{ACPSH}$$

Such an assay enabled independent study of two significant and separate events, internal acylation of a protein and characterization of a toxin. The enzyme was fully characterized and evidence that HlyC functions as an acyltransferase is presented. Because Ca^{2+} is required for toxin function, the effects of Ca^{2+} on HlyA acylation were studied. The kinetic parameters of acyl-ACPs bearing diverse acyl groups were

determined as well as the toxicities of the acyl-toxins they produced. Notably, HlyC formed a reactive acyl-enzyme intermediate, and the acyl-HlyC intermediate was able to catalyze the reverse reaction transferring the acyl group to ACPSH forming acyl-ACP.

The kinetic or chemical mechanism of an enzyme catalyzing the internal acylation of a protein has never been determined. In the present study, the kinetic mechanism of the internal acylation of proHlyA catalyzed by HlyC was determined by steady state kinetics and by reaction product inhibition studies. Finally, chemical modification and site-directed mutagenesis of HlyC were used as tools to determine residues involved in the catalysis of the acyl transfer reaction contributing to the delineation of the chemical mechanism.

CHAPTER 2

MATERIALS AND METHODS

Biologicals and Reagents

[1-^{14}C]labeled fatty acids, [1-^{14}C] palmitoyl-CoA, and [1-^{14}C]sodium pantothenate were from New England Nuclear except for lauric acid which was from Amersham. American Radiolabeled Chemicals, Inc. supplied [9,10-^{3}H]labeled palmitoleic acid. Palmitoyl-CoA was from P.L. Biochemicals. Novagen was the source of alkaline phosphatase conjugated S-peptide. Nitrocellulose, 0.45 μM, was from Costar. Spectrum Medical Industries, Inc. was the source of Spectra/Gel A4. Agar for DNA electrophoresis was from FMC. The following items were supplied by Fisher Scientific: 40% stock acrylamide:bisacrylamide (37.5:1) solution, hydroxylamine (NH_2OH), dithiothreitol (DTT), isopropyl thiogalactoside (IPTG), sodium dodecyl sulfate (SDS), sodium deoxycholate, urea, chloramphenicol, kanamycin, tetracycline, ampicillin, bovine serum albumin, Spectra/Por dialysis tubing, bacto-tryptone, bacto-yeast extract and agar. N-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), phenylmethylsulfonyl fluoride (PMSF), 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF), diethylpyrocarbonate (DEPC), tetranitromethane (TNM), phenylglyoxal (PG), thrombin, ribonuclease S-protein, Kodak film, dimethyl suberimidate, Coomassie brilliant blue R, and Tween-20 were from Sigma. *Eco*RV, deoxynucleotides, ammonium persulfate, 5-bromo-4-chloro-3-indoyl phosphate (BCIP), nitroblue tetrazolium (NBT), N, N, N', N',-tetramethylethylenediamine (TEMED), Wizard DNA Clean-Up System, Wizard Miniprep and Midiprep DNA Purification System were from Promega. New England Biolabs was the source of T4

DNA ligase, calf intestinal phosphatase, Deep Vent DNA polymerase, Deep Vent 10X buffer, and *Dpn* I. *Pfu Turbo* DNA polymerase and XL-2 Blue Ultracompetent cells were from Stratagene. Protein molecular weight standards were from Bio-Rad, Novex, and New England Biolabs. High and low DNA mass standards were from Life Technologies. All chemicals were reagent grade.

Escherichia coli

Novagen was the source of *E. coli* strains HMS174, BLR(DE3), BLR(DE3)pLysS, BL21(DE3)pLysS, and NovaBlue cells. Permanent stocks of *E. coli* were stored at –80 °C as 8% glycerol stocks. The genotype of each strain is listed in Table 1. *E. coli* cultures were routinely grown in Luria broth (LB) (*54*) at 37 °C at 200 rpm. Other bacterial growth media included M9 minimal media (*54*), 2XTY and TB medium (*55*). LB-agar plates were prepared by the addition of 20 g agar per liter LB medium. Growth media were supplemented with either kanamycin, tetracycline, chloramphenicol, or ampicillin with final concentrations of 34 μg/mL, 12.5 μg/mL, 34 μg/mL and 50 μg/mL respectively.

Recombinant DNAs and Techniques

Preparation of Competent *E. coli* Cells

All bacterial strains used for the introduction of plasmid DNA were made competent by the calcium chloride method of transformation (*56*), except for the XL-2 Blue strain which was purchased from Stratagene.

Table 1. *E. COLI* STRAIN GENOTYPE DESCRIPTIONS

Strain	Genotype
HMS174	F^- recA hsdR ($r_{K12}^- m_{K12}^-$) Rif^R
BLR(DE3)	F^- ompT $hsdS_B$ ($r_B^- m_B^-$) gal dcm Δ(srl-recA)306::Tn10 (Tc^r) λ (DE3)
BLR(DE3)pLysS	F^- ompT $hsdS_B$ ($r_B^- m_B^-$) gal dcm Δ(srl-recA)306::Tn10 (Tc^r) λ (DE3) pLysS (Cm^r)
BL21(DE3)pLysS	F^- ompT $hsdS_B$ ($r_B^- m_B^-$) gal dcm λ(DE3) pLysS (Cm^r)
XL-2 Blue strain	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F' proAB $lacI^qZ\Delta M15$ Tn10 (Tet^r) Amy Cam^r]^a
NovaBlue	endA1 hsdR17 ($r_{K12}^- m_{K12}^+$) supE44 thi-1 recA1 gyrA96 relA1 lac[F' $proA^+B^+$ $lacI^qZ\Delta M15$::Tn10 (Tc^r)]

Transformation

The method for the introduction of plasmid DNA into *E. coli* host, except for the XL-2 Blue strain, was that outlined by the pET System Manual from Novagen (*55*). The method of transformation of XL-2 Blue *E. coli* was that outlined by the Epicurian Coli Ultracompetent and Supercompetent Instruction Manual from Stratagene. XL-2 Blue *E. coli* were only used for transforming PCR products prepared for the construction of mutant *hlyCs*.

Plasmid Purification

Plasmids containing the native *hlyC* or mutant *hlyC* genes were propagated in NovaBlue and XL-2 Blue *E. coli* strains respectively. The Wizard Miniprep and Midiprep DNA purification systems were used for the small-scale and large-scale isolations of plasmid respectively. These systems were used only when the resulting plasmid

preparation was needed as template for DNA sequencing reactions, template for site-directed mutagenesis, or during vector preparation for subcloning. Isolation of plasmid DNAs from positive clones during subcloning of hemolysin genes were performed following a method outlined by the pET system manual from Novagen (*55*).

DNA Agarose Gel Electrophoresis

DNA electrophoresis was carried out using the Minicell EC370 electrophoretic gel system from Fisher Scientific. For routine analysis of DNA samples, samples were run on 40 mM Tris-acetate, 1 mM EDTA (TAE) agarose gels and stained during electrophoresis by including ethidium bromide in the TAE electrophoresis buffer at 0.33 μg/mL. DNA samples were mixed with 50% glycerol solution containing 0.25% w/v bromophenol blue prior to application to the gel. Electrophoresis was routinely performed at 100 V and monitored by movement of the bromophenol blue. Excess ethidium bromide was removed by destaining in water for 20 minutes. The presence of DNA was visualized by UV-transillumination on a 312 nm variable intensity transilluminator. For preparation of DNA to be isolated from agarose gel slices for subsequent applications, no ethidium bromide was included in either the agarose gel or the electrophoresis buffer. To locate DNA to be isolated from the gel, an additional lane containing a duplicate sample was separated from the rest of the gel and stained for 20 min in TAE buffer containing ethidium bromide as described above.

DNA Quantification

DNA was quantified by comparison to either low DNA (100 bp - 2000 bp) mass or high DNA (1000 bp - 10,000 bp) mass standards electrophoresed along with samples of unknown concentrations.

Genetic Materials

Novagen was the source of pET plasmids. Dr. Colin Hughes provided the construct pEK50 (*57*) containing the *hlyCABD* operon used to produce native HlyA. Both pTXA1 and pTXC1 were prepared by Dr. Lesa Worsham in this laboratory and served as separate hyperexpression systems for proHlyA and S-tag-HlyC respectively (*58*).

Oligonucelotides

Oligonucleotides used for subcloning of the native *hlyC* gene, site-directed mutagenesis of *hlyC*, or for colony screening were purchased from Integrated DNA Technologies and are described in Table 2.

Construction of Recombinant His_6-S-tag-HlyC

HlyC was expressed as a His_6-S-tag fusion protein using pET30(a)+ and a 513 bp PCR product from pEK50 encoding HlyC. pET30a(+) vector treated with the restriction endonuclease *Eco*RV, followed by treatment with calf intestinal phosphatase, was separated from remaining undigested pET30a(+) using a 0.7% agarose gel. Gel slices containing the digested vector were removed and the DNA isolated using a spin column. Vector was concentrated by precipitation with ethanol and sodium acetate (*55*, *59*). The

Table 2: OLIGONULCEOTIDES

Name[a]	Sequence (5' to 3')[b]
HlyC Upper	ATGAATATAAACAAACCATTAGAGATTCTT
HlyC Lower	TTAACCAGTTAATGAAAAATTAAAATCTGA
T7 Promoter	TAATACGACTCACTATAGGG
G11A Upper	CCATTAGAGATTCTTGCTCATGTATCCTGGCTATGGGCCAG
G11A Lower	CTGGCCCATAGCCAGGATACATGAGCAAGAATCTCTAATGG
S20A Upper	CCTGGCTATGGGCCAGTGCTCCACTACACAGAAACTGGCC
S20A Lower	GGCCAGTTTCTGTGTAGTGGAGCACTGGCCCATAGCCAGG
H23A Upper	GGCTATGGGCCAGTTCTCCACTAGCTAGAAACTGGCCAGTATC
H23A Lower	GATACTGGCCAGTTTCTAGCTAGTGGAGAACTGGCCCATAGCC
H23C Upper	GGCTATGGGCCAGTTCTCCACTATGTAGAAACTGGCCAGTATC
H23C Lower	GATACTGGCCAGTTTCTACATAGTGGAGAACTGGCCCATAGCC
H23S Upper	GGCTATGGGCCAGTTCTCCACTATCTAGAAACTGGCCAGTATC
H23S Lower	GATACTGGCCAGTTTCTAGATAGTGGAGAACTGGCCCATAGCC
R24A Upper	GGGCCAGTTCTCCACTACACGCTAACTGGCCAGTATCTTTG
R24A Lower	CAAAGATACTGGCCAGTTAGCGTGTAGTGGAGAACTGGCCC
C57A Upper	GATTACCCTGTCGCGTATGCTAGTTGGGCTAATTTAAGTTTAG
C57A Lower	CTAAACTTAAATTAGCCCAACTAGCATACGCGACAGGGTAATC
S58A Upper	GATTACCCTGTCGCGTATTGTGCTTGGGCTAATTTAAGTTTAG
S58A Lower	CTAAACTTAAATTAGCCCAAGCACAATACGCGACAGGGTAATC
E67A Upper	GGGCTAATTTAAGTTTAGAAAATGCTATTAAATATCTTAATGATG
E67A Lower	CATCATTAAGATATTTAATAGCATTTTCTAAACTTAAATTAGCCC
Y70G Upper	GTTTAGAAAATGAAATTAAAGGTCTTAATGATGTTACCTCATTAG
Y70G Lower	CTAATGAGGTAACATCATTAAGACCTTTAATTTCATTTTCTAAAC
Y70F Upper	GTTTAGAAAATGAAATTAAATTCCTTAATGATGTTACCTCATTAG
Y70F Lower	CTAATGAGGTAACATCATTAAGGAATTTAATTTCATTTTCTAAAC
S76A Upper	CTTAATGATGTTACCGCTTTAGTTGCAGAAGACTGGACTTCAGG
S76A Lower	CCTGAAGTCCAGTCTTCTGCAACTAAAGCGGTAACATCATTAAG
G85A Upper	GCAGAAGACTGGACTTCAGCTGATCGTAAATGGTTCATTGAC
G85A Lower	GTCAATGAACCATTTACGATCAGCTGAAGTCCAGTCTTCTGC
D86A Upper	CAGAAGACTGGACTTCAGGTGCTCGTAAATGGTTCATTGACTGG
D86A Lower	CCAGTCAATGAACCATTTACGAGCACCTGAAGTCCAGTCTTCTG
R87A Upper	CAGAAGACTGGACTTCAGGTGATGCTAAATGGTTCATTGACTGG
R87A Lower	CCAGTCAATGAACCATTTAGCATCACCTGAAGTCCAGTCTTCTG
Y150G Upper	GCGAATAAAATTTTTAAACAAGGTCACCACGAGTTAATAACTG
Y150G Lower	CAGTTATTAACTCGTGGTGACCTTGTTTAAAAATTTTATTCGC
Y150F Upper	GCGAATAAAATTTTTAAACAATTCCACCACGAGTTAATAACTGAAG
Y150F Lower	CTTCAGTTATTAACTCGTGGTGGAATTGTTTAAAAATTTTATTCGC

[a]Letters refer to one amino acid code. [b]Codons shown in bold encode amino acid changes.

DNA encoding for *hlyC* was prepared by PCR reactions (100 μL) containing 50 ng of pEK50 plasmid DNA as template, 10 μL of 10X Deep Vent Polymerase buffer, 4 mM Mg^{+2}, 300 μM deoxynucleotides, 0.5 μM of phosphorylated HlyC upper and HlyC lower primers, and 2 U of Deep Vent Polymerase. The following PCR program was used: (i) 1 cycle with 2 min at 95 °C, 58 °C for 45 sec, and 30 sec at 72 °C; (ii) 30 cycles with 45 sec at 95 °C, 45 sec at 58 °C, and 30 sec at 72 °C; and (iii) 1 cycle with 45 sec at 95 °C, 58 °C for 45 sec, and 5 min at 72 °C. PCR was performed using a GTC-2 GeneticThermal Cycler. Following amplification, the homogenous 513 bp product was processed through a Promega DNA Clean-Up Kit to remove unwanted polymerase and buffer contents.

The *hlyC* PCR product and vector were combined at a 1:3 ratio (vector:PCR product) and ligated using 2,000,000 units ligase per 20 μL reaction at 16 °C for 16 h. The ligation reaction was transformed into NovaBlue cells and cells were screened for the presence and proper orientation of the *hlyC* gene using colony PCR (*55*). Colony PCR reactions (20 μL) contained colony lysate as template, 2 μL of 10X Deep Vent Polymerase buffer, 4 mM Mg^{+2}, 300 μM deoxynucleotides, 0.5 μM of HlyC Lower and T7 promoter primer, and 0.4 U of Deep Vent Polymerase. The following PCR program was used: (i) 1 cycle with 2 min at 95 °C, 58 °C for 45 sec, and 43 sec at 72 °C; (ii) 30 cycles with 45 sec at 95 °C, 45 sec at 58 °C, and 43 sec at 72 °C; and (iii) 1 cycle with 45 sec at 95 °C, 58 °C for 45 sec, and 5 min at 72 °C. Positive screens gave a 700 bp PCR product. Plasmid DNA from positive colonies was transformed into BL21(DE3)pLysS cells for protein expression. The plasmid encoding for His_6-S-tag-HlyC fusion protein was designated pTXC2.

Construction of Mutant HlyCs

Site-directed mutations in *hlyC* were generated by a round circle PCR-based mutagenesis procedure (*60*) using the plasmid pTXC2 as the reaction template. Using one upper and lower primer for each mutation described in Table 2 led to the replication of both plasmid strands with the desired mutation. For example, upper and lower H23A primers were used to change His 23 to an alanine. The PCR reactions (50 µL) contained 50 ng of pTXC2 plasmid DNA, 5 µL of 10X *Pfu Turbo* polymerase buffer, 200 µM deoxynucleotides, 2.5 U of *Pfu Turbo* DNA polymerase, and 0.2 µM of one upper and lower primer for the desired mutation listed in Table 2. The following PCR program was used: (i) 1 cycle with 2 min at 95 °C, 55 °C for 1 min, and 12 min at 72 °C; (ii) 15 cycles with 45 sec at 95 °C, 55 °C for 1 min, and 12 min at 72 °C; and (iii) 1 cycle with 45 sec at 95 °C, 55 °C for 1 min and 15 min at 72 °C. Following amplification of the mutated pTXC2 plasmid, 20 U of the *dam* methylation specific restriction enzyme, *Dpn* I, was added to each amplification reaction and incubated at 37 °C for 1.5 hrs to digest the parental, methylated (nonmutated template), supercoiled DNA. PCR reactions were applied to a 1% agarose gel, and the presence of a PCR product of ~6 kb was confirmed. To ensure complete digestion of the parental plasmid, a negative control PCR reaction containing everything but the mutagenic primers was also subjected to *Dpn I* digestion. PCR reactions and negative controls were transformed into XL-2 Blue Ultracompetent cells from Stratagene. Individual colonies were screened, and plasmid DNAs from positive colonies transformed into BL21(DE3) pLysS cells. Recombinant DNA plasmids

were designated pTXC2 with the mutation described afterward. For example, pTXC2 H23A encodes for a His_6-S-tag-HlyC with His 23 changed to alanine.

DNA Sequencing

Samples were sequenced using the T7 promoter primer except for the Y150F and Y150G mutants. For these constructs the HlyC upper primer was used in the sequencing reactions. Sequencing was done at the Molecular Genetics Facility at the University of Georgia on an ABI 373 Stretch DNA Sequencer using the TAQ Dye terminator chemistry method (*61*, *62*).

Protein Expression and Purifications

Expression and Purification of ProHlyA

ProHlyA was isolated from inclusion bodies in BLR(DE3)pLysS or BL21(DE3) pLysS cells containing pTXA1. Cells (200 mL culture) were grown in LB at 37 °C, induced with 1 mM IPTG at $A_{600\ nm}$ 0.6, and harvested 2 h later. Subsequent steps were at 4 °C. Cells were harvested at 10,000 × *g* for 10 min, resuspended in 5 ml of HED buffer [25 mM HEPES (pH 8.0), 5 mM EDTA, 2 mM DTT] and stored at –20 °C. Upon thawing, cells were exposed briefly to ultrasound. Inclusion bodies were collected by centrifugation at 20,000 × *g* for 20 min and washed with 25 mL portions of the following solutions: 0.5% sodium deoxycholate in HED buffer; twice with 0.1% sodium deoxycholate in HED buffer; and finally three times with HED buffer without deoxycholate. ProHlyA was solubilized in 0.6 mL of HED buffer containing 6 M urea for 1.5 h. Following centrifugation at 30,000 × *g* for 30 min, the clarified solution was

divided into aliquots and stored at –20 °C. Urea-containing buffers were always freshly prepared.

Secreted proHlyA was obtained from the supernatant solution of a 1.0 L BLR(DE3)/pTXA1 culture. Cells were removed by centrifugation. ProHlyA was precipitated at 65% $(NH_4)_2SO_4$ saturation and stirred for 16 h at 4 °C. After centrifugation at 25,000 × g for 30 min, the precipitate was washed with 0.2 M citrate (pH 3.0). The precipitate was dissolved in 3.0 mL of HED buffer containing 6 M urea and stored in aliquots at –20 °C.

Expression and Purification of HlyCs

Cells (200 mL culture) for HlyC expression were grown in minimal media at 37 °C, induced with 1 mM IPTG at $A_{600\ nm}$ 0.6, harvested 4-5 h later, resuspended, and stored in 5 mL of HED buffer at –20 °C. HlyC was expressed as an S-tag or His_6-S-tag fusion protein using pTXC1 or pTXC2 respectively. The fusion protein was isolated at 4 °C generally from inclusion bodies or occasionally from the soluble portion of cells. Thawed cells were briefly exposed to ultrasound. Soluble fusion protein was obtained from the cell lysate supernatant by centrifugation at 100,000 × *g* for 1 h and stored at –20 °C. Alternatively, inclusion bodies from either pTXC1 or pTXC2 cells were isolated from the cell lysate pellet (200 mL culture) collected by centrifugation at 20,000 × *g* for 20 min. Inclusion bodies were washed three times with 25 mL portions of HED buffer. The HlyC was solubilized by suspending the inclusion bodies in 15 mL of HED 6 M urea buffer for 1.5 h. The fusion protein solution was clarified by centrifugation at 30,000 × *g* for 30 min. The solution was processed one of several ways:

First, protein from inclusion bodies was refolded by stepwise removal of urea by dialysis (8 M_r cutoff). The protein solution in 6 M urea was dialyzed against 1.0 L of HED 3 M and 1 M urea buffers, each for 3 h, and finally against HED buffer for 16 h. The solution was clarified by centrifugation at 30,000 × *g* for 30 min. Fusion protein was precipitated overnight at 65% $(NH_4)_2SO_4$ saturation, collected by centrifugation at 30,000 × g for 30 min, and dissolved in HED buffer. Protein was 2 mg.

To obtain highly purified S-tag-HlyC fusion protein from BL21(DE3)pLysS/ pTXC1 cells, an S-protein affinity medium was used (*63*, *64*). Fusion protein, 2 mg, was incubated overnight in 25 mM HEPES (pH 8.0), 0.15 M NaCl, 0.01% Triton X-100, 2 M urea with 1 mL of Spectra/Gel A4 which contained S-protein affinity ligand. The medium was washed with 50 mL of the same buffer followed by 30 mL 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 3.0), 2 M urea and dialyzed against 25 mM MES (pH 6.1), 2 M urea followed by HED 2 M urea buffer. Finally, it was freed of urea by dialysis against HED buffer and clarified by centrifugation at 30,000 × *g* for 30 min.

To purify the His_6-S-tag-HlyC, the protein was bound to an affinity His-Bind metal chelation resin containing immobilized Ni^{2+} ions. His_6-S-tag-HlyC was isolated from inclusion bodies from 500 mL of BL21(DE3)pLysS/pTXC2 cells as described above. 15 mg of protein in 7.5 mL of 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 5 mM imidazole, 6 M urea was applied to a 1.5 mL column of His-Bind resin at a flow rate of 10 column volumes/h. The column was washed with 20 mL of 20 mM Tris-HCl (pH 7.9), 0.5 M NaCl, 5 mM imidazole, 6 M urea followed by 20 mL of 20 mM Tris-HCl (pH 7.9), 0.5 M

NaCl, 20 mM imidazole, 6 M urea or until the $A_{280\ nm}$ was <0.01. His_6-S-tag-HlyC was eluted with 20 mM Tris (pH 7.9), 0.5 M NaCl, 500 mM imidazole, 6 M urea. Urea was removed by stepwise dialysis as described above and the solution clarified by centrifugation at 30,000 × *g* for 30 min. The purified His_6-S-tag-HlyC was stored in aliquots at –20 °C.

Expression and Purification of HlyA

HlyA was obtained from the supernatant solution of a culture of HMS174 containing the plasmid pEK50 encoding for the complete *hlyCABD* operon. Cells were grown in LB to $A_{600\ nm}$ 1.0 and centrifuged 10,000 × *g* for 10 min at 4 °C. Subsequent handling was at 4 °C. The solution was filtered through a 0.45 μm filter, and the filtrate was brought to 65% saturation with ammonium sulfate. The precipitate was processed like that described for secreted proHlyA. HlyA was freshly prepared for each group of experiments and the preparations checked for viability at the time of use by erythrocyte lysis (described below).

Expression and Purification of Acyl-ACPs

Radiolabelled acyl-ACPs were prepared using radioactive fatty acids. Acyl-ACPs were prepared by Dr. Lesa Worsham from *E. coli* ACPSH and acyl-ACP synthase (*65*, *66*, *67*) as described by Trent and colleagues (*58*).

Expression and Purification of [1-^{14}C]ACPSH

ACPSH was isotopically labeled by adding 2 μM [1-^{14}C]sodium pantothenate (0.6 μCi) (*68*, *69*) to 50 mL cultures of *E. coli* grown and processed to purify ACPSH as described (*58*). The purified ACPSH had a specific radioactivity of 0.3 Ci/Mol and was prepared by Dr. Lesa Worsham.

Protein Concentration

Protein concentrations were measured by the Bradford assay (*70*).

Protein Gel Electrophoresis, Western Blotting, and Fluorography

SDS-PAGE followed the procedure of Laemmli (*71*). A Bio-Rad Semi-Dry Electrophoretic Transfer Cell was used according to the manufacturer's instructions for Western Blots. Protein was transferred onto 0.45 μm nitrocellulose for 30 min at 15 V. Autofluor from Midwest Scientific was used for fluorography. Gels were dried according to the manufacturer's instructions using the Bio-Rad Gel Dryer and exposed to BioMax MR film using a Kodak Biomax Transcreen-LE Intensifying Screen.

Assay of Acyltransferase Activity

The assay was done in microfuge tubes in a total volume of 100 μL in 100 mM HEPES (pH 8.0), 2 μM [^{14}C]acyl-ACP, 22 μg 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). The urea concentration did not exceed 0.4 M. Reaction was started by the addition of [^{14}C]acyl-ACP. Following a 5 min incubation at 4 °C, reaction was stopped by placing the tubes in an 80 °C bath for twenty min. Enzyme was inactivated immediately; however,

longer incubation at 80 °C was shown to enhance the precipitation of the [^{14}C]acyl-HlyA. 233 μL of saturated $(NH_4)_2SO_4$ in 500 mM Tris (pH 7.0) was added to achieve 70% saturation. After 30 min at 4 °C, precipitated protein was collected by centrifugation at 13,600 × *g* for 5 min. Acyl-ACP does not precipitate under these conditions. The pellet was washed and vortexed with 500 μL 50% isopropanol, 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.

In some cases, ~2 μM native and mutant HlyCs were assayed by a quick dilution method. For these reactions, enzyme isolated from inclusion bodies was immediately diluted in 25 mM Hepes (pH 8.0) until the urea concentration was 0.4 M. The protein was incubated on ice for 25 min, followed by the addition of the substrates, proHlyA and [^{14}C]acyl-ACP, and assayed as described above.

Kinetic Mechanism of HlyC

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 (*72*) which calculated kinetic parameters by several different methods and by the nonlinear regression analysis of Wilkinson (*73*); the different methods of computing kinetic constants agreed with R values greater than 0.96. or else the data were rejected. Reported kinetic constants for the different acyl-ACP

substrates were those computed from the Direct Linear analyses (*74*) using the nonlinear regression analysis of Wilkinson (*73*).

Steady State Kinetics and Product Inhibition Studies

Reaction velocities and kinetic data were determined as described above. The different methods of computing kinetic constants agreed giving R values greater than 0.97 except for product inhibition experiments that contained both proHlyA and HlyA. At higher concentrations proHlyA and HlyA have a tendency to aggregate (*75*, *76*), and R values in these circumstances ranged from 0.91 to 1. Description of kinetic data plots are given in the individual figure legends.

Product inhibition studies were performed as described above except for those containing HlyA. Assays in which HlyA product inhibition was measured contained 1.5 M urea, in order to discourage aggregation of HlyA; this concentration of urea had no detectable effect on the other proteins in the assay.

Chemical Cross-Linking of Proteins Involved in ProHlyA Acylation and Acyl-Enzyme Intermediate Formation

The reaction was set up as described above for assay of acyltransferase activity with the cross linking reagent, dimethyl suberimidate (DMS), added last. Any variations in substrates are given in Figure legends 2, 32, and 33. The reaction mixes were exposed to 10 mM DMS for 10 min at 25 °C. Chemical cross-linking was 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 13,600 × *g* for 5 min, washed with 500 µL acetone, and the pellet collected by centrifugation. Protein was dissolved in 20 µL 8 M urea + 20 µL 2 % SDS sample buffer and subjected to SDS-PAGE followed by fluorography. For acyl-enzyme intermediate formation for both native and mutant HlyCs, protein mixes were incubated for 10 min at 4 °C without DMS. Protein was precipitated with trichloroacetic acid and processed as described above.

Assay for the Formation of Acyl-[^{14}C]ACPSH from Acyl-HlyC and [^{14}C]ACPSH

acyl-ACP + HlyC ↔ [HlyC • acyl-ACP] ↔ acyl-HlyC + ACPSH

The assay for the transfer of a long chain fatty acyl group from the enzyme intermediate, acyl-HlyC, to radiolabeled ACPSH as described by the equation above was as follows: Using the HlyC assay conditions described above, unlabeled myristoyl-ACP, 25 µM , was incubated with either 25 or 50 µM HlyC for 10 min at 4 °C in order to form the putative reaction intermediate, unlabeled acyl-HlyC. The reverse reaction was begun by the addition of 25 µM [^{14}C]ACPSH followed by a 10 min incubation at 4 °C. Following reaction, samples were made 10% in trichloroacetic acid and kept on ice for 45 min then centrifuged at 13,600 × *g* for 5 min. The protein pellet was washed with 500 µL of cold acetone, dissolved in 25 µL 8 M urea + 20 µL 2% SDS sample buffer, and subjected to 15% SDS-PAGE followed by fluorography. Control reactions, which were processed identically, included the following combinations: 25 µM myristoyl-ACP + 25 µM [^{14}C] labeled ACPSH; 50 µM HlyC + 25 µM [^{14}C] labeled ACPSH; and 25 µM [^{14}C] labeled ACPSH.

pH Activity Studies

Acyltransferase acitvity was assayed at a variety of pHs as described above using a Mes, Hepes, Tris, Glycine buffer system. Buffers were mixed together and used as a single buffer system titrated to the desired pH. All buffers in the assay were at a final concentration of 100 mM and NaCl was added to maintain an ionic strength of 0.52 M.

Chemical Modification of HlyC

Chemical modifications of HlyC with a variety of group specific reagents were carried out primarily according to published procedures (*77*). The modification reactions were intitated by the addition of freshly prepared chemical reagents in a particular solvent to S-tag-HlyC isolated from inclusion bodies as described above. For each modification reaction, a control reaction was subjected to the same treatment with solvent only. All results were expressed as the percentage of the control activity. For each chemical modification reaction, the reagent was either removed by physical means or the reaction quenched as described below. Controls were done to ensure that the chemical modifier was completely removed before assay.

Modification by N-ethylmaleimide (NEM), *p*-Chloromercuribenzoate (PCMB)

A 260 mM solution of NEM was prepared in water and the concentration determined by measuring the $A_{302\ nm}$ using an extinction coefficient of 620 $M^{-1}cm^{-1}$ (*78*). A 1 mL protein solution (2.5 mg) in 0.1 M KPO_4 (pH 7.0) buffer containing S-tag-HlyC was treated with 1.0 mM NEM at 25 °C for 15 min. Excess reagent was removed by application of the reaction to a 10 mL Sephadex G-25 fine column equilibrated in 25 mM

Hepes (pH 8.0) buffer. Protein was eluted with 25 mM Hepes (pH 8.0) buffer containing 0.1 M NaCl. Fractions were measured for $A_{280\ nm}$ and a portion of the peak fraction (~10 μg protein) assayed for acyltransferase activity.

A stock solution of PCMB was prepared by dissolving the solid in 0.1 M Tris (pH 11.0) buffer, followed by centrifugation at 30,000 × *g* for 30 min to remove insoluble material. A 10 mM PCMB working solution was prepared by diluting a portion of the stock solution in 0.01 M KPO_4 (pH 7.0) buffer. The concentration of the PCMB solution was determined by measuring the $A_{233\ nm}$ using an extinction coefficient of 1.69×10^4 $M^{-1}cm^{-1}$ (*78*). 100 μL of a 2.1 μg/μL HlyC solution in 20 mM Mes (pH 6.0) buffer was exposed to 1.0 mM PCMB by addition of a portion of a 10 mM PCMB solution. Reaction proceeded for 10 min at 25 °C, and the excess reagent was removed using a centricon with a 5,000 MW cut-off. Following washing with 300 μl of HED buffer, 10 μg of modified protein was assayed for acyltransferase activity.

Modification by Phenylmethylsulfonyl Fluroide (PMSF) and 4-(2-Aminoethyl)-benzenesulfonyl Fluoride (AEBSF)

Ten mM solutions of PMSF and AEBSF were prepared by dissolving the solids in dry isopropanol and dimethyl sulfoxide respectively. For both inhibitors, a 1.37 μg/μL HlyC solution in 100 μL of 25 mM Hepes (pH 8.0) was modified by 1.0 mM of the reagent for 10 min at 25 °C. Following reaction, the reagents were removed using a centricon with a 5,000 MW cut-off and the sample washed with 300 μl of HED buffer. Ten μg of protein was assayed for acyltransferase activity.

Modification by Diethylpyrocarbonate (DEPC)

A 65 mM DEPC stock solution was prepared in acetonitrile. The concentration of the reagent was determined by reaction with 10 mM imidazole in 0.1 M KPO_4 (pH 6.0) buffer and measuring the increase in A_{240nm} using an extinction coefficient of 3200 $M^{-1}cm^{-1}$ for the modified imidazole derivative (*79*). A 22 µL 2.0 µg/µL HlyC solution in 0.1 M KPO_4 (pH 6.0 or pH 8.0) buffer was exposed to various amounts of DEPC at 25 °C for 10 min and the reaction quenched by the addition of a 20-fold molar excess of imidazole in 0.1 M KPO_4 (pH 6.0 or pH 8.0) buffer. To measure the modification reaction over time, a larger reaction was prepared, and at timed intervals, 10 µg aliquots containing enzyme were removed, quenched, and assayed. The presence of imidazole had no effect of acyltransferase activity.

Hydroxylamine (NH_2OH) Treatment of Modified HlyC

Following treatment with 2 mM DEPC and quenching as described above, 2 M NH_2OH (pH 7.0) was added to a final concentration of 100 mM. At various time intervals, 10 µg aliquots of enzyme were removed and assayed as described above. Activity was expressed as the percentage of a control HlyC sample (10 µg) treated with acetonitrile and 100 mM NH_2OH for 240 min.

Modification by Tetranitromethane (TNM)

Modification of HlyC by TNM was carried out by the addition of various amounts of an ethanol solution of the reagent (10 mM) to a 100 µL solution of enzyme at 0.95 µg/µL in 0.05 M Tris (pH 8.0) or 0.1 M KPO_4 (pH 6.0) buffers at 25 °C. Reactions were

quenched by the addition of a 20-fold molar excess of L-tyrosine in 0.05 M Tris (pH 8.0) buffer and 10 μg of modified enzyme assayed as described above. To measure the modification reaction over time, a larger reaction was prepared, and at timed intervals, 10 μg aliquots of enzyme were removed, quenched, and assayed. The presence of excess tyrosine had no effect on acyltransferase activity.

Modification with N-acetylimidazole (NAI)

NAI was prepared from acetic anhydride (100 mmol) and imdiazole (87 mmol) as described by Staab and Rohr (*80*). The product was dissolved in warm acetone and precipitated with the addition of petroleum ether and the crystals dried and stored dessicated at –20 °C. Concentration of the reagent in acetonitrile was determined spectrophotometrically at 240 nm using an extinction coefficient of 3200 M^{-1} cm^{-1}.

Chemical modification of HlyC by NAI was carried out by the addition of a freshly prepared acetonitrile solution of the reagent (100 mM) to a 50 μL 0.2 μg/μL enzyme solution in 50 mM Hepes (pH 7.5). The reaction was performed for 30 min at 25 °C. Following modification, the reagent was removed by 70% $(NH_4)_2SO_4$ precipitation and the enzyme dissolved in 25mM Hepes (pH 7.0) buffer. 10.0 μg of modified HlyC was measured for acyltransferase activity at a pH of 7.0.

Modification with Phenylglyoxal (PG)

A 200 mM solution of PG was prepared in 25 mM Hepes (pH 8.0) containing 50% ethanol. The reagent was added to a 50 μL, 0.2 μg/μL, HlyC solution in 25 mM Hepes (pH 8.0) buffer at 25 °C for one hour. Reactions were quenched by the addition of 20-

fold molar excess of L-arginine in 25 mM Hepes (pH 8.0) and 10 μg of modified protein assayed for acyltransferase activity. Excess arginine had no effect HlyC activity.

Substrate/Product Protection Studies

Chemical modification protection experiments were performed by incubating 10 μg of enzyme solution in 100 μl of HED buffer for 5 min with either 2 μM unlabeled myristoyl-ACP or ACPSH followed by chemical modification by one of the reagents described above. Enzyme was treated with either 2 mM DEPC, 5 mM PG, or 50 fold molar excess of TNM (0.22 mM). Prior to assay, samples were precipitated at 70% $(NH_4)_2SO_4$ saturation at pH 7.0 at 4 °C and the enzyme was collected by centrifugation. Neither acyl-ACP nor ACPSH precipitates under these conditions, therefore any species of ACP were removed. The enzyme was then assayed using [1-^{14}C]myristoyl-ACP as described above.

Spectral Studies

Modification of HlyC by DEPC or TNM was monitored spectrally using a HP 8452A Diode-Array Spectrophotometer at wavelengths ranging from 200-500 nm at 2 nm increments. For both reactions, a double sectored cuvette was used with the enzyme solution placed in the first sector and the modifying reagent placed in the second. For DEPC modification, a 1.0 mg/mL HlyC solution in 0.1 M KPO_4 (pH 6.0) was modified with 1.0 mM DEPC at 25 °C. A spectrum was taken before mixing of the two solutions that served as the blank. Reactions were initiated by mixing the contents of the two sectors by inversion of the cuvette and spectra taken at various time intervals. For study

of spectral changes attributed to TNM modification, a 2.72 mg/mL HlyC solution in 0.05 M Tris (pH 8.0) was modified with a 50-fold molar excess of TNM (6.0 mM) at 25 °C. A spectrum was taken before mixing and one 30 min after initiation of reaction. For both spectra, 0.05 M Tris (pH 8.0) buffer served as the blank.

Characterization of HlyC Mutants

Mutant HlyCs were purified from inclusion bodies as described above. Mutants were characterized like the native HlyC by performing chemical cross-linking reactions, detection of acyl-enzyme intermediate, and determination of K_m and V_{max} for both proHlyA and myristoyl-ACP substrates as described above.

Assessment of HlyA Lytic Capability

HlyA as an agent of erythrocyte lysis was measured in 0.05% suspensions of erythrocytes as described (*81*). Assays of proHlyA activation were done as described above except the 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. In order to compare lytic capabilities of the separate acyl-HlyA's, 20 μL aliquots of enzyme assay reaction mixtures immediately after completion and cessation of reaction, each containing a different acyl-ACP, were assayed for erythrocyte lysis.

CHAPTER 3

RESULTS

Expression and Purification of Proteins

ProHlyA was expressed using recombinant DNA (pTXA1) containing the *hlyABD* genes. Expression of proHlyA was independent of different growth conditions. Figure 2 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, since pTXA1 encodes 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). The protein was stored in 6 M urea and was stable at -20 °C for greater than 6 months. Each liter of culture yielded ~10-12 mg of proHlyA from inclusion bodies and 1.5 mg of proHlyA from the cell culture media.

HlyC was either expressed as an S-tag or His_6-S-tag fusion protein using pTXC1 or pTXC2 respectively. The highest yields of HlyC were obtained from cultures grown in minimal media at 37 °C harvested five hours after induction with 1 mM IPTG. Approximately, 60 mg of HlyC was expressed per liter of cell culture. The S-tag-HlyC fusion protein was seen in SDS-PAGE of the total cell lysate as an intensely staining band at M_r 22,500 (Figure 3A 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

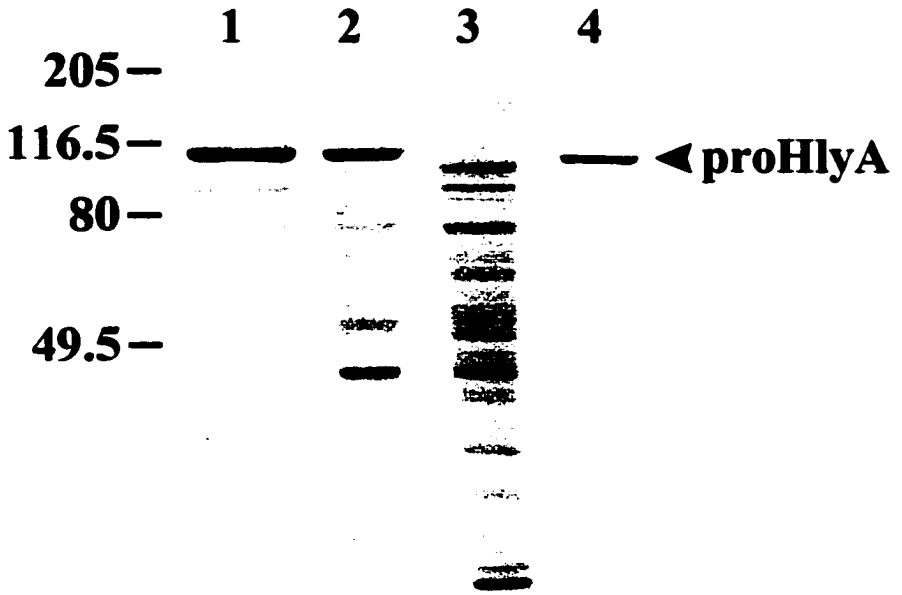


Figure 2: ProHlyA expression and purification. An SDS-10% PAGE showing distribution and purification of proHlyA. The arrow marks the position of the 107 kDa proHlyA. The gel was loaded as follows: lane 1, 10 μg of protein from the BLR(DE3)pTXA1 cell growth supernatant solution; lane 2, 10 μg of the BLR(DE3)pLysS/pTXA1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

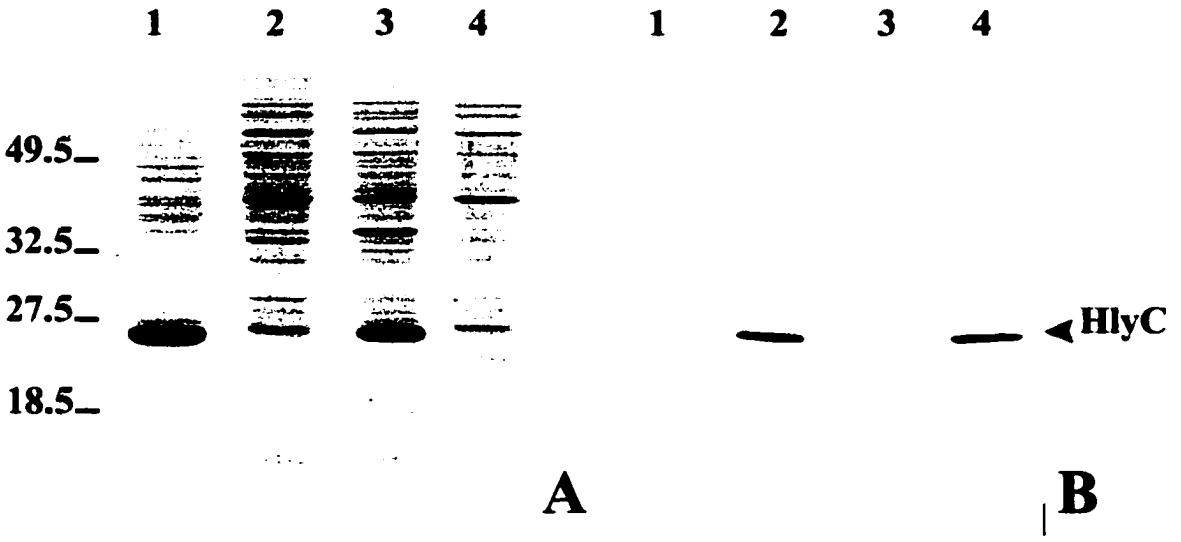


Figure 3: Expression of S-tag-HlyC. An SDS-15% PAGE (A) and its western blot probed with S-protein alkaline phosphatase conjugate (B) showing the cellular distribution of S-tag-HlyC fusion protein. Lanes in A contained 10 μg of protein from the following sources: lane 1, insoluble fraction of cell lysate; lane 2, soluble fraction of the cell lysate; lane 3, cell lysate; lane 4, lysate of cells without pTXC1. The gel in 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. The arrow shows the position of the fusion protein at 22.5 kDa for both the stained gel and western blot.

of cells lacking the recombinant DNA (lane 4) contained none. A Western blot of the separated proteins (Figure 3B) 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 (not shown). The two dark bands (lanes 2 and 4) of the western blot (Figure 3B) confirmed the identity of the intensely stained Coomassie Blue bands (Figure 3A, lanes 1 and 3) as S-tag-HlyC fusion protein. Densitometric analysis indicated that >50% of the insoluble protein was the fusion protein. Cellular location and expression of the His_6-S-tag-HlyC from pTXC2 was identical to that of S-tag-HlyC from pTXC1. HlyC activity was assayed as described subsequently; sources of active enzyme included cell lysates, the 100,000 × *g* solution of the cell lysate, and urea extracts of washed inclusion bodies.

Characterization of HlyC-Dependent ProHlyA Activation

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 1,500-3,000 dpm were obtained routinely. Assay blanks which contained heat-denatured HlyC measured about 50 counts/min. HlyC source was 50 μg 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 nanomoles fatty acyl group transferred/min/mg protein where HlyC was ~0.1% of the protein. In the absence of the recombinant *hlyC* or its expression, there was no proHlyA-acyl-ACP acyltransferase activity.

Fluorography of SDS-PAGE of the assay precipitates (Figure 4) 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.

To verify that HlyC was responsible for the acyltransferase activity, both the S-tag-HlyC and the His_6-S-tag-HlyC fusion protein were solublized from washed inclusion bodies (Figure 5A lane 3 and Figure 5B lane 2) in buffered 6 M urea and affinity purified. S-tag-HlyC was applied to S-peptide-liganded Sepharose 4A. Following washing, elution at pH 3.0, and renaturation by dialysis to HED buffer the fusion protein was seen as a single band at Mr 22,500 in Figure 5A, lane 4. ~0.4 mg/L of culture of pure S-tag-HlyC was obtained by this method. His_6-S-tag-HlyC from inclusion bodies (Figure 5B lane 2) was applied to an affinity His-Bind metal chelation resin containing immobilized Ni^{2+} ions. Following washing and elution with 500 mM imidazole, the His_6-S-tag-HlyC

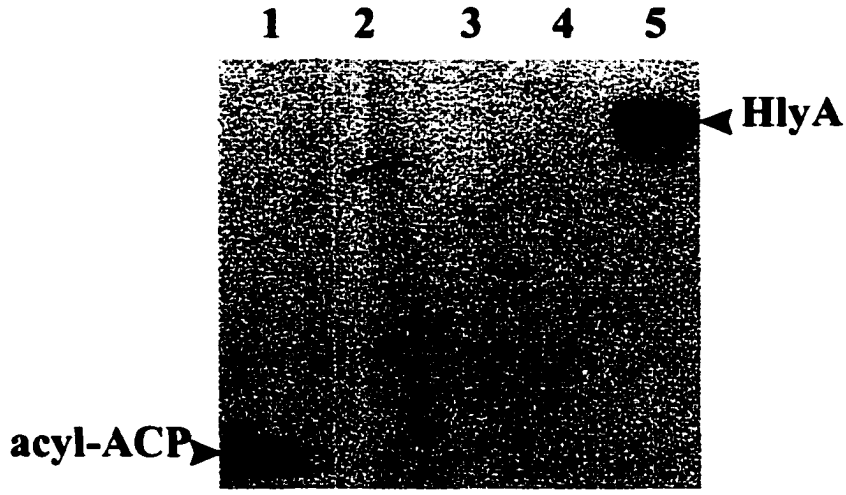


Figure 4: Dependence of acyl-ACP-proHlyA acyl transfer on HlyC. Fluorography of an SDS-12% PAGE of redissolved assay precipitates showing the transfer of radiolabeled fatty acid from acyl-ACP (M_r ~9,000) to proHlyA forming radiolabeled HlyA (M_r 107,000). The method was that of Worsham and colleagues (*82*), 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 25 mM HEPES (pH 8.0), 1 mM DTT, 5 mM EDTA, 5 M urea and treated with 100 µl 2 M NH_2OH (pH 10.0) for 30 min at 37 °C. Following precipitation with cold trichloroacetic acid, samples were dissolved in SDS-PAGE sample buffer. [1-^{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.

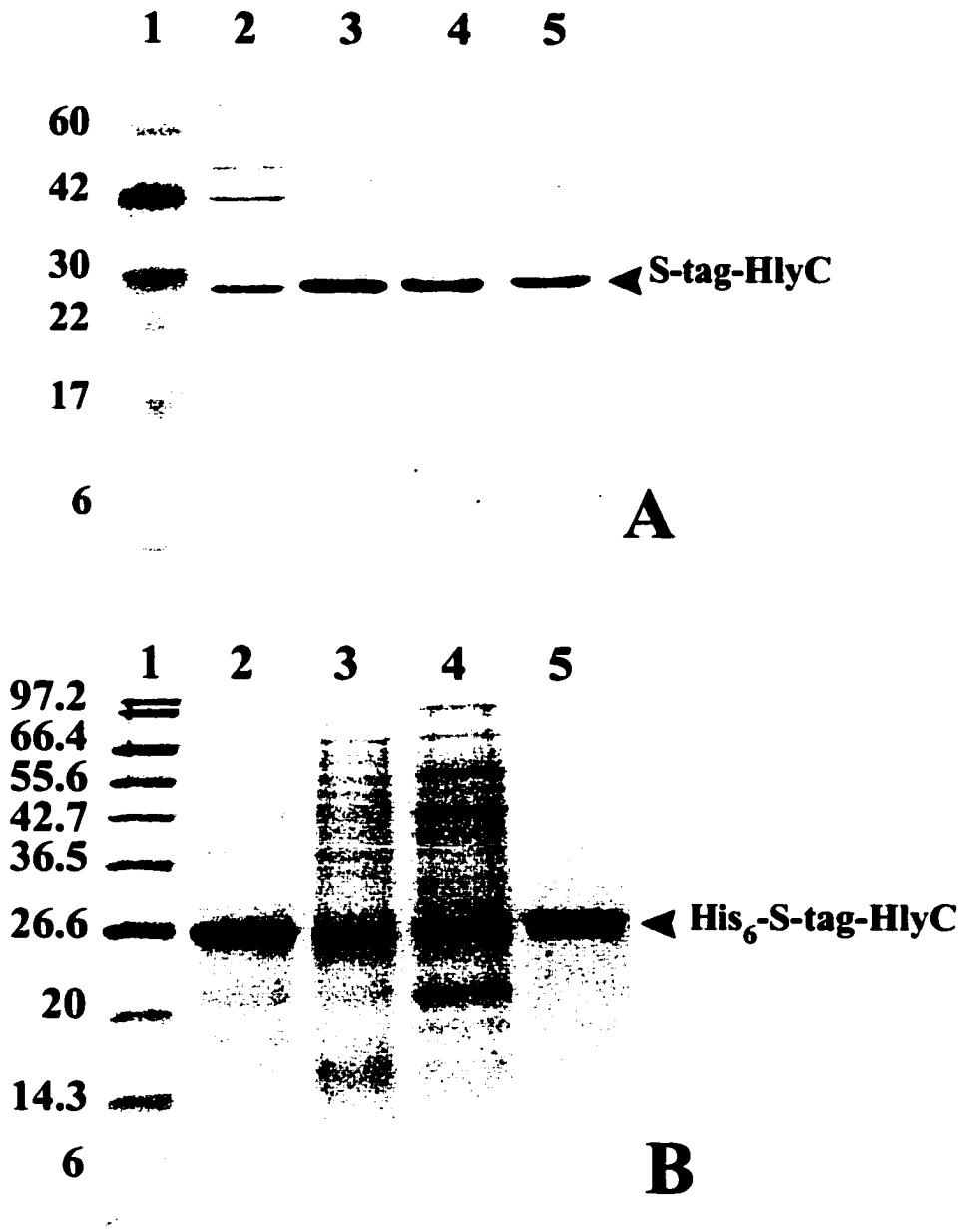


Figure 5: Purification of S-tag- (A) and (B) His_6-S-tag-HlyC. (A) SDS-15% PAGE illustrating the S-tag-HlyC purification as described in the text. Proteins from the

Figure 5 (continued): 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 3.0 eluate from S-protein affinity chromatography; lane 5, TSK-250 HPLC (Figure 7) peak fraction of acyl-ACP-proHlyA acyltransferase activity. (B) SDS-15% PAGE illustrating the His_6-S-tag-HlyC purification as described in the text. Proteins from the following sources were loaded in 10 μg aliquots: lane 1, mass standards in kDa; lane 2, washed inclusion bodies; lane 3, flow through; lane 4, 20 mM imidazole wash; lane 5, 500 mM imidazole eluate from the His-Bind metal chelation resin.

fusion protein electrophoresed at a Mr of 23,000 as shown Figure 5B lane 5. The yield of pure HlyC by this method was ~8 mg/L of culture. The purified HlyC from the imidazole elution was renatured by dialysis against HED buffer. The pure His_6-S-tag- and S-tag-HlyCs both possessed acyl-ACP-proHlyA acyltransferase activity. The activities and stabilities of these preparations is discussed below.

The fused tags attached to the N-terminus of HlyC did not influence acyltransferase activity. The acyl-ACP-proHlyA acyltransferase activities of two aliquots of soluble S-tag-HlyC from 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). S-tag or His_6-S-tag-HlyC fusion proteins and HlyC resulting from thrombin cleavage showed the same amount of acyltransferase activity.

A demonstration of the thrombin cleavage is shown in Figure 6. An S-peptide probe of an SDS-PAGE western blot shows the purified S-tag-HlyC (Figure 6B, 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 kD molecular weight marker is thrombin. The 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).

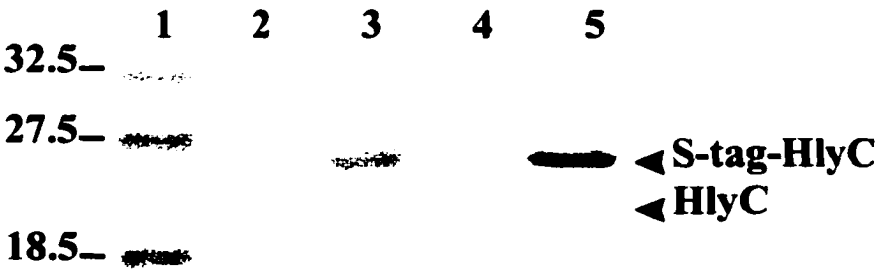


Figure 6: Thrombin Cleavage of S-tag-HlyC. SDS-15% PAGE and Western blot showing thrombin cleavage of S-tag-HlyC fusion protein. Proteins in lanes 1-3 were stained with Coomassie Blue, and proteins in lanes 4 and 5 were Western-blotted and probed with S-peptide-alkaline phosphatase conjugate. Lane 1, 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 treatment. Lanes 4 and 5 were each loaded with 1 μg of fusion protein after and before exposure to thrombin, respectively.

Table 3. COMPARISON OF HLYC ACYLTRANSFERASE ACTIVITIES

Source of HlyC	Specific Activity*
100,000 × g supernatant of cell lysate	200,000
Urea Extracts of Washed Inclusion Bodies	502
Eluate from S-Protein affinity resin (Pure S-tag-HlyC)	278
Eluate from His-bind resin (Pure His_6-S-tag-HlyC)	1100

*units are pmol of fatty acyl group transferred/min/mg protein

Although the eluate from the S-protein affinity resin resulted in a highly purified and active fusion protein, the relatively harsh conditions of purification and renaturation lowered the specific activity compared to that of soluble HlyC which had never been in inclusion bodies nor exposed to urea. A more active preparation of HlyC from 6 M urea was obtained by step wise removal of the denaturant by dialysis. The specific activity of the resulting enzyme solution was 502 pmol of acyl group transferred/min/mg of protein where HlyC was estimated as >50% of the total protein. This preparation was used routinely for studies of the enzyme. Purified His_6-S-tag-HlyC resulted in a preparation of similar activity (Table 3).

The size of the active form of HlyC was shown by size exclusion HPLC to be ~22,500 M_r where fraction 29 showed the highest activty (Figure 7). Thus HlyC was enzymatically active as a monomer. The largest $A_{280\ nm}$ peak in Figure 7, fraction 38, was DTT in the sample applied. SDS-PAGE of the protein from the fraction which

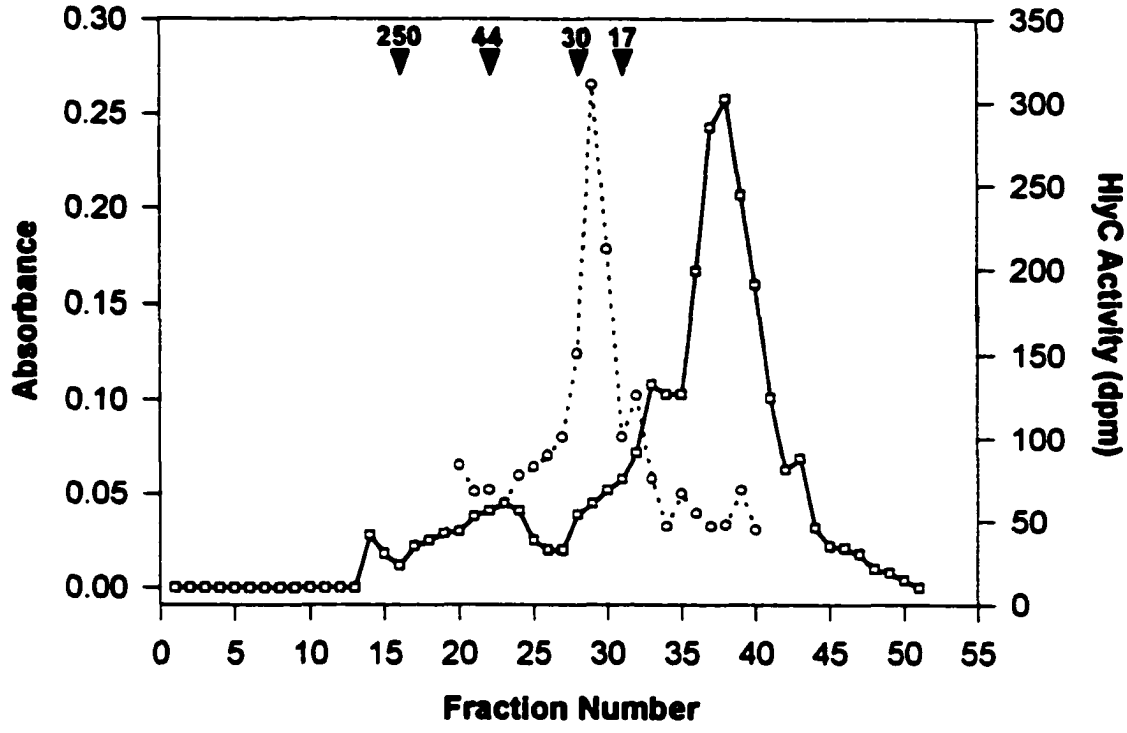


Figure 7: Size exclusion HPLC showing M_r of acyl-ACP-proHlyA acyltransferase activity. 0.3 mg of S-tag-HlyC in 25 mM HEPES (pH 8.0), 5 mM EGTA, 1 mM DTT, 0.3 M urea, extracted from inclusion bodies was applied to a TSK-250 column (Bio-Rad) on a Waters model 510 HPLC. Column and elution buffer was 25 mM HEPES (pH 7.2), 0.05 M NaCl. Fractions of 0.5 ml were collected, measured for A_{280} (□) and acyl-ACP-proHlyA acyltransferase activity (o). Protein from the peak fraction of enzyme activity was analyzed by SDS-PAGE, Figure 5A lane 5. Arrows denote elution peaks of calibration proteins of the indicated sizes in kDa.

contained the highest acyltransferase activity (Figure 5A lane 5) showed a single protein, the size of HlyC.

The reaction rate was linear with respect to HlyC concentration (Figure 8), and the amount of product formed was linear with time at 4 °C (Figure 9). Michaelis-Menten kinetics were observed. With myristoyl-ACP held constant at 1 μM, proHlyA had a K_m^{app} of 0.94±0.1μM and V_{max}^{app} 7.5±0.2, and myristoyl-ACP had a K_m^{app} of 0.48±0.1 μM, and a V_{max}^{app} of 6.9±0.5 with proHlyA held constant at 2 μM. Diverse acyl-ACPs served as substrates, but when [1-^{14}C]palmitoyl-CoA was substituted for radiolabeled acyl-ACP, there was no incorporation of radioactivity into proHlyA. 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 (Figure 10). The acyltransferase activity was relatively stable. It remained constant indefinitely at 4° C or -20° C, but at 25 °C for 16 h about one half of the activity survived. Enzyme was destroyed quickly at higher temperatures. Several freeze-thaw transitions were tolerated with no diminution of activity.

The pH dependency of the enzyme catalyzed acylation of proHlyA was measured over a range of pH at constant ionic strength (Figure 11). Acyltransferase activity was undetectable below pH 6.0 and increased sharply between pH 6.5 and 7.5, with maximal activity seen at pH 9-10. Activity fell sharply at pH>10.0. The formation of the

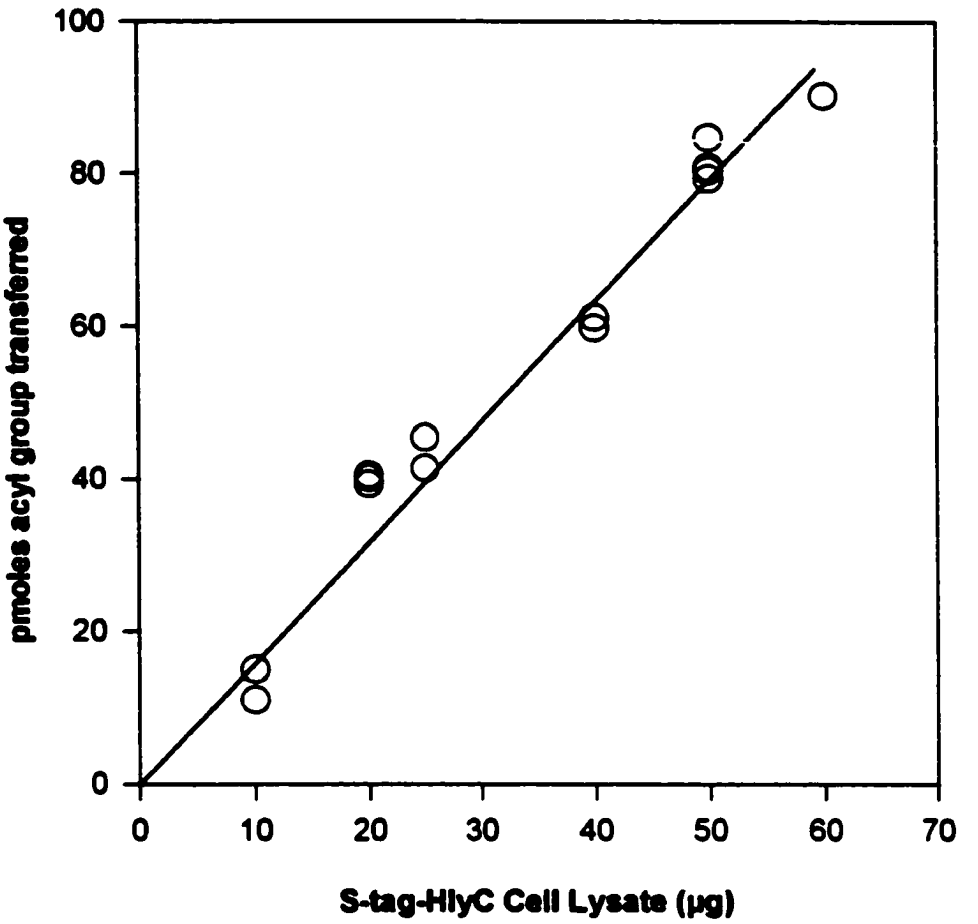


Figure 8: Dependence of acyl-ACP-proHlyA acyl transfer reaction on HlyC. Assays were done as described in Materials and Methods in the presence of increasing amounts of cell lysate which contained S-tag-HlyC fusion protein.

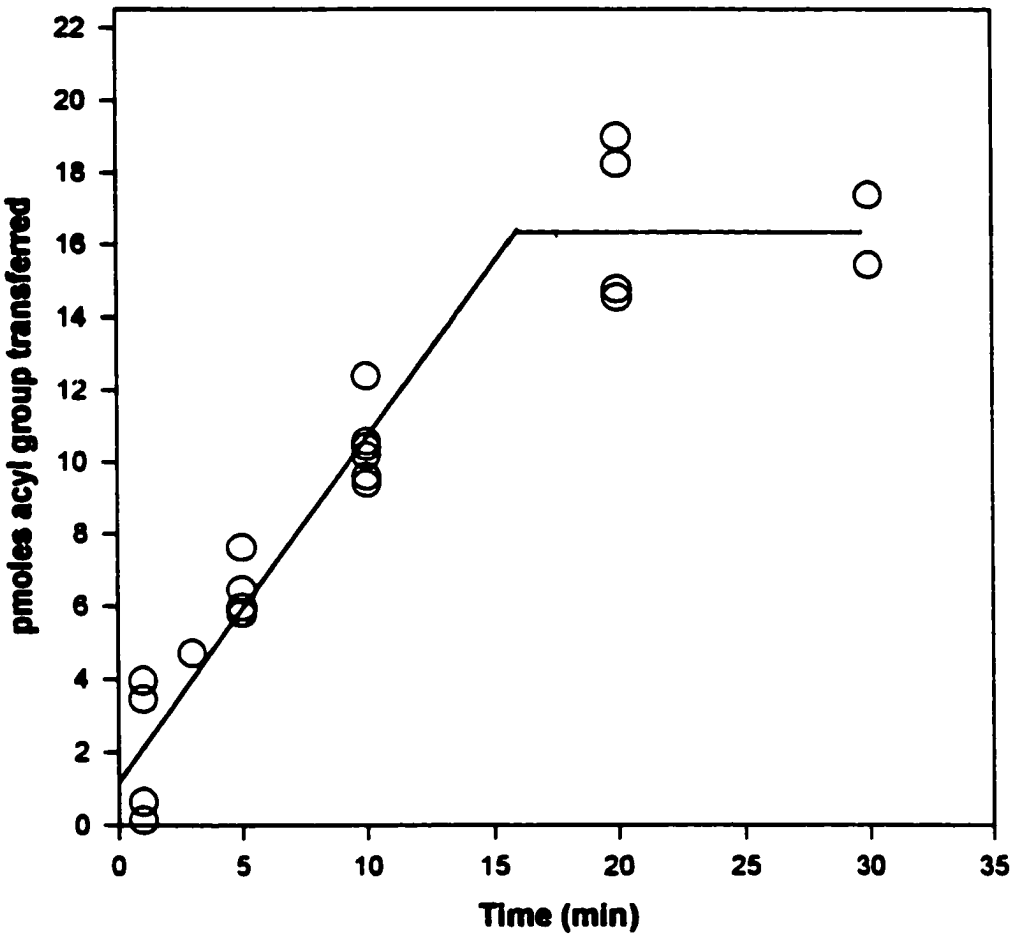


Figure 9: HlyC acyltransferase activity as a function of time. Assays were done as described in Materials and Methods at various time intervals.

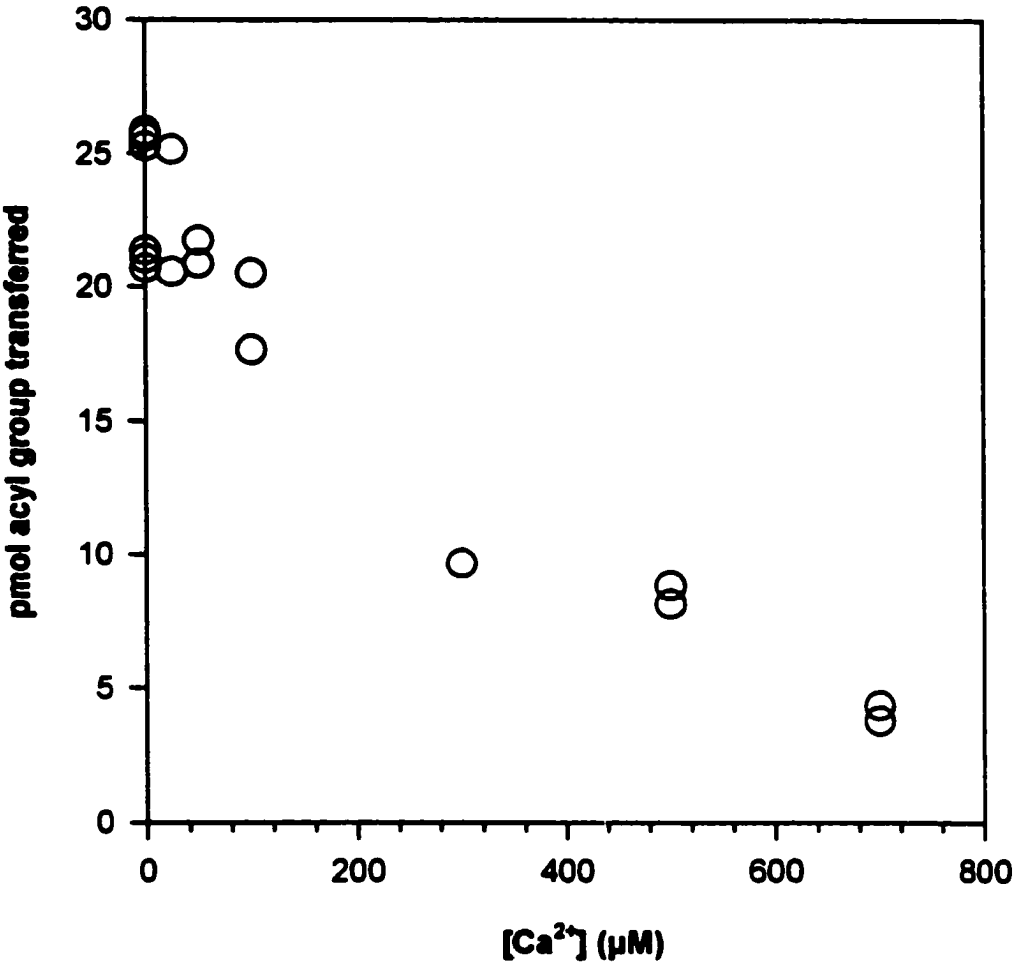


Figure 10: Effects of Ca^{2+} on HlyC acyltransferase activity. The assay was carried out as described in Materials and Methods except that increasing amounts of Ca^{2+} were added.

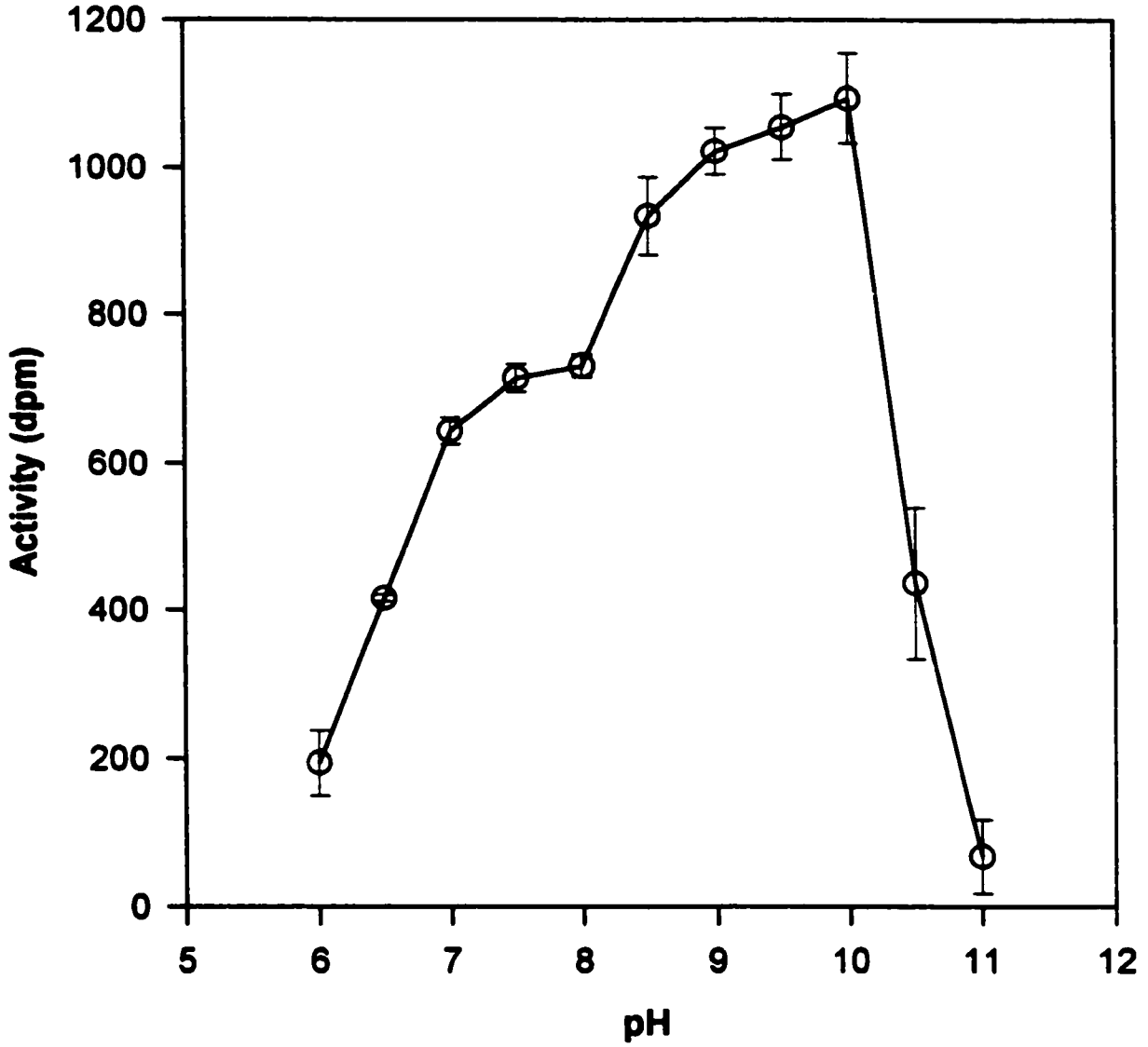


Figure 11: Effects of pH on HlyC acyltransferase activity at constant ionic strength. Assays were performed at different pH as described in Materials and Methods.

radiolabelled product, HlyA, at various pHs was also examined by fluorography of redissolved assay precipitates and showed the same trend with pH (data not shown).

Chemical cross-linking of proHlyA, [^{14}C]acyl-ACP, and HlyC in different combinations followed by SDS-PAGE and fluorography resulted in visualization of discrete protein-protein complexes (Figure 12). Dimethylsuberimidate (DMS) treatment of solutions which contained all three 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 DMS (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 were formed (lane 4). When a solution of HlyC and [^{14}C]acyl-ACP was prepared with no exposure to DMS 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

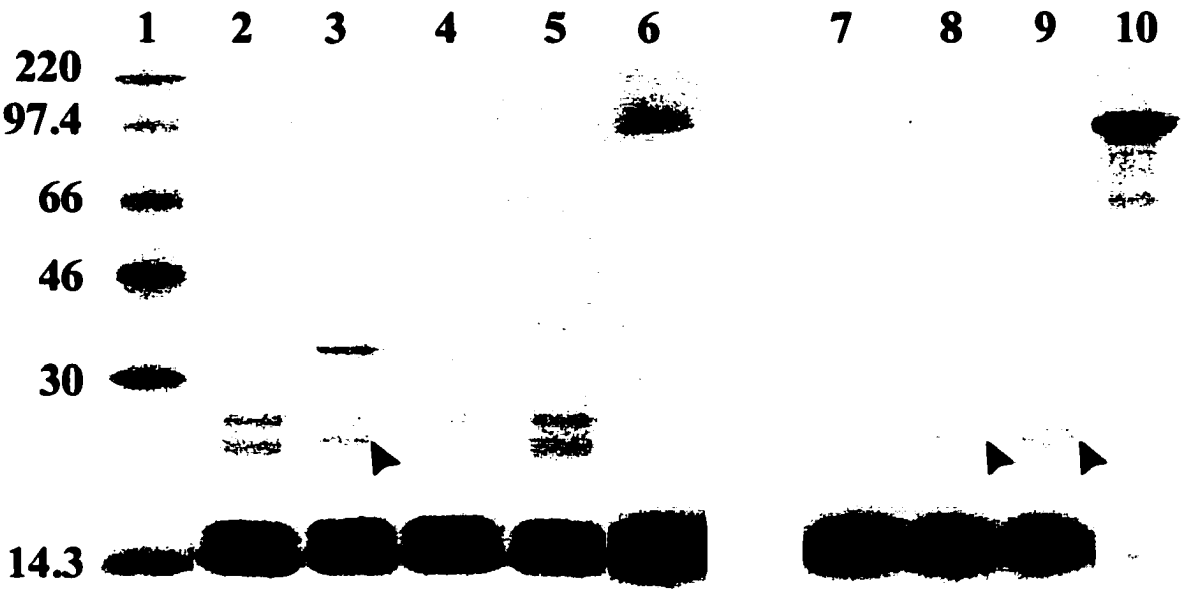


Figure 12: Fluorographic demonstration of acyltransferase reaction proteins which cross-linked in the presence of DMS (dimethylsuberimidate) and the formation of an acyl-enzyme intermediate (arrow) with and without DMS treatment. Lane 1 contained [^{14}C]labeled mass standards in kDa. Lanes 2 through 6 contained combinations of acyltransferase reaction proteins exposed to DMS, subjected to SDS 12% PAGE, and fluorograped as described (*82*). Kodak BioMax MR film was used. Each assay treated with DMS 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

Figure 12 (continued): min at 4 °C and were not exposed to DMS. 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 in Materials and Methods.

portion while proHlyA was added to the other. Both solutions were processed like the other protein solutions in Figure 12. 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).

The demonstration of a reactive covalent acyl-enyzme intermediate indicated that the acyltransferase reaction likely proceeded by two partial reactions as follows:

1) acyl-ACP + HlyC ↔ [HlyC • acyl-ACP] ↔ acyl-HlyC + ACPSH

2) acyl-HlyC + proHlyA ↔ [proHlyA • acyl-HlyC] ↔ HlyA + HlyC

sum) acyl-ACP + proHlyA ↔ HlyA + ACPSH

Another means of demonstrating the occurrence of the acyl-enzyme would be to show the reversibility of partial reaction 1. Radioactively labeled ACPSH was made by purifying ACPSH from *E. coli* grown in the presence of [1-^{14}C]pantothenate which labels the prosthetic group covalently attached to the protein (*68*, *69*). Unlabeled myristoyl-ACP and HlyC were mixed in an assay reaction and incubated for 10 min at 4 °C in order to allow formation of unlabeled acyl-HlyC, one of the two substrates in the reverse of partial reaction 1. Then radiolabeled ACPSH, the second substrate in the reverse of partial reaction 1, was added, and after another 10 min incubation at 4 °C, the proteins were precipitated, redissolved, and separated by SDS-PAGE. The formation of radiolabeled acyl-ACP from unlabeled acyl-HlyC and radiolabeled ACPSH is shown in Figure 13 lanes 6 and 7. Twice as much HlyC was used to generate acyl-HlyC in the reaction analyzed in lane 7 compared to that analyzed in lane 6, and a larger amount of

myristoyl-ACP was evident on the fluorogram. An assay that contained only acyl-ACP and labeled ACPSH (lane 5) showed no labeled acyl-ACP so detectable amounts of uncatalyzed acyl group exchange did not occur under the conditions employed. An assay that contained only HlyC and labeled ACPSH showed no radioactivity at the position of acyl-ACP (not shown). Thus HlyC in the form of acyl-HlyC catalyzed the proposed partial reaction 1 in the reverse direction converting ACPSH to acyl-ACP.

Characterization of Diverse Acyl-ACP Substrates Compared with Erythrocyte Lysis by the Different Acyl-Toxins

The kinetic parameters of acyl-ACP's containing different acyl groups were determined (Table 4). 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 4. 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 4). Increasing chain

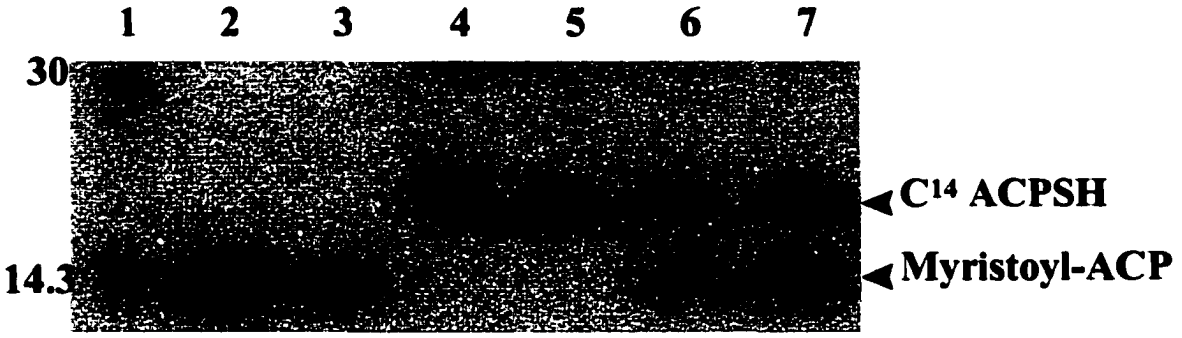


Figure 13: Fluorographic demonstration of the reversibility of partial reaction 1. Protein mixtures were prepared, treated, analyzed by 15% SDS-PAGE, and subjected to fluorography as described in Experimental Procedures. The following proteins were applied to lanes 1-3: lane 1, radioactive mass standards in kDA; lane 2, [^{14}C]myristoyl-ACP, 20 Ci/Mol, 0.5 μg; and lane 3, [^{14}C]myristoyl-ACP, 20 Ci/Mol, 0.25 μg . Reaction mixtures of 100 μL containing the indicated proteins prepared and processed as described above were analyzed in the following lanes: lane 4, 25 μM [^{14}C]ACPSH; lane 5, 25 μM [^{14}C]ACPSH + 25 μM myristoyl-ACP; lane 6, 25 μM myristoyl-ACP + 25 μM HlyC followed by 25 μM [^{14}C]ACPSH; lane 7, 25 μM myristoyl-ACP + 50 μM HlyC followed by 25 μM [^{14}C]ACPSH. The [^{14}C]ACPSH was 0.3 Ci/Mol.

Table 4: KINETIC PARAMETERS OF DIFFERENT ACYL-ACP'S IN THE ACYLTRANSFERASE REACTION[a]

acyl-ACP	acyl chain	K_m^{app}	% of myristoyl-ACP K_m^{app}	V_{max}^{app}	% of myristoyl-ACP V_{max}^{app}	V_{max}^{app}/K_m^{app}
lauroyl-	12:0	0.46±0.1	96	5.7±0.2	83	12.3
myristoyl-	14:0	0.48±0.1	100	6.9±0.5	100	14.3
palmitoyl-	16:0	0.96±0.1	200	8.5±0.2	124	8.9
palmitoleoyl-	$16:1^{\Delta 9}$	0.91±0.2	190	2.2±0.2	33	2.5
stearoyl-	18:0	8.5±0.5	1771	7.0±0.1	102	0.8
oleoyl-	$18:1^{\Delta 9}$	6.4±0.6	1325	5.9±0.3	87	0.9

[a] K_m^{app} is μM, and V_{max}^{app} is pmol acyl group 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.

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 with increasing chain length. There was not much difference between oleoyl- and stearoyl-ACP's, 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 the carbon 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 5). Acyltransferase assays each contained an identical amount of a different acyl-ACP donor, and, following reaction, each had formed different amounts of HlyA as predicted by Table 5, column 3. If toxicity was independent of the nature of the acyl group on the toxin, hemolysis (Table 5, column 5) should parallel toxin formation (column 3), which it did 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. The most effective acyl-ACP substrate, myristoyl-ACP, produced product, myristoyl-HlyA, that caused half of the lysis that stearoyl-HlyA caused, but stearoyl-ACP-supported toxin activation produced 94% less acyl-toxin than did that employing myristoyl-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.

Table 5: COMPARISON OF SUBSTRATE EFFICACIES OF ACYL-ACP'S WITH LYTIC ABILITIES OF THE CORRESPONDING ACYL-HLYA'S

acyl-ACP	acyl chain	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-	12:0	12.3	0.739±0.004	0.6
myristoyl-	14:0	14.3	0.438±0.038	0.3
palmitoyl-	16:0	8.9	0.614±0.012	0.7
palmitoleoyl-	$16:1^{\Delta 9}$	2.5	0.451±0.005	1.8
stearoyl-	18:0	0.8	0.785±0.069	9.5
oleoyl-	$18:1^{\Delta 9}$	0.9	0.813±0.112	8.7

[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 acyltransferase 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} 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 4) by the corresponding proportionality constant for the acyltransferase activity of that particular acyl-ACP (column 3).

Kinetic Mechanism of HlyC

Steady State Kinetics of HlyC

Since myristoyl-ACP was the most effective substrate for the acyltransferase (Table 4), it was used for determination of the steady state kinetic mechanism of HlyC. The two-substrate reaction catalyzed by HlyC shown below,

$$\text{proHlyA} + \text{acyl-ACP} \xrightarrow{\text{HlyC}} \text{HlyA} + \text{ACPSH}$$

likely proceeds by way of one of two possible reaction mechanisms which are summarized as follows. One entails formation of a substituted enzyme intermediate where one product is released prior to the binding of the second substrate; alternatively, a ternary complex is formed where both substrates combine with the enzyme in either a random or compulsory order prior to the release of any product. Steady state kinetics was done to distinguish between the two general mechanisms. Measurements of initial reaction velocities at varying concentrations of one substrate at several fixed concentrations of the other substrate yielded data that in double-reciprocal and half-reciprocal plots formed patterns that distinguished between a substituted enzyme or ternary complex mechanism. Initial reaction rates of proHlyA acylation were determined by varying myristoyl-ACP concentrations at different fixed concentrations of proHlyA (Figure 14) and by varying proHlyA concentrations at different fixed myristoyl-ACP concentrations (Figure 15). In both instances, the graphs showed a series of parallel lines on double reciprocal plots (Figures 14A and 15A) and common intersection of all linear plots on the [S]/v axis for half-reciprocal plots (Figures 14B and 15B). Each type of plot

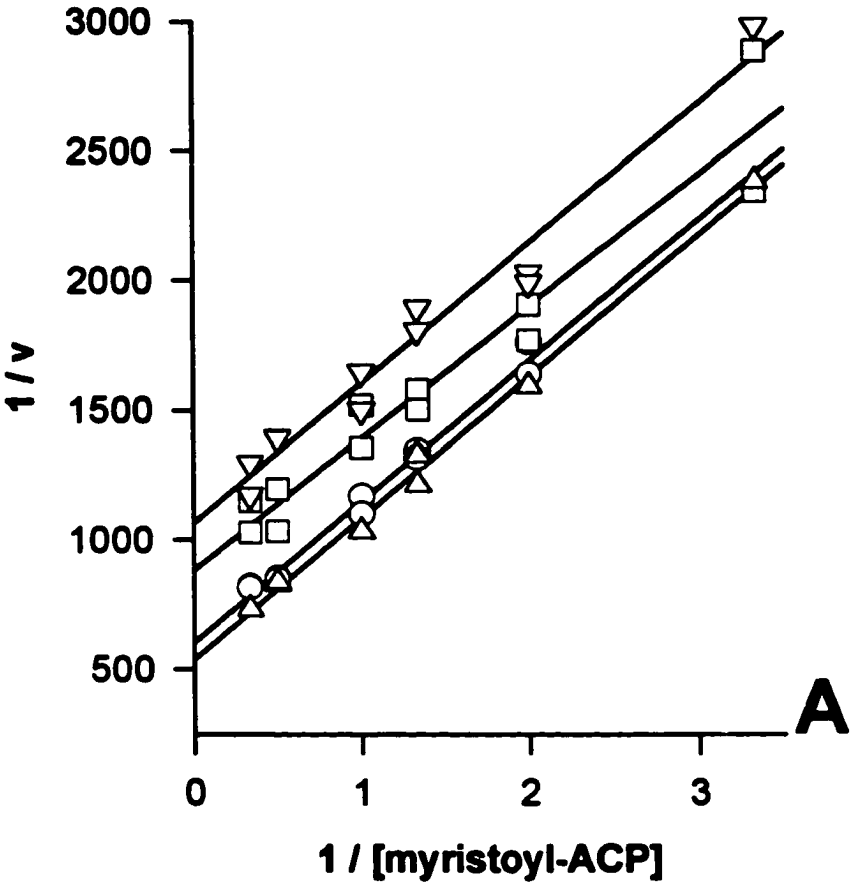


Figure 14: Myristoyl-ACP initial velocity data at different fixed non-saturating concentrations of proHlyA shown as double reciprocal plots (A) and as half-reciprocal plots (B).

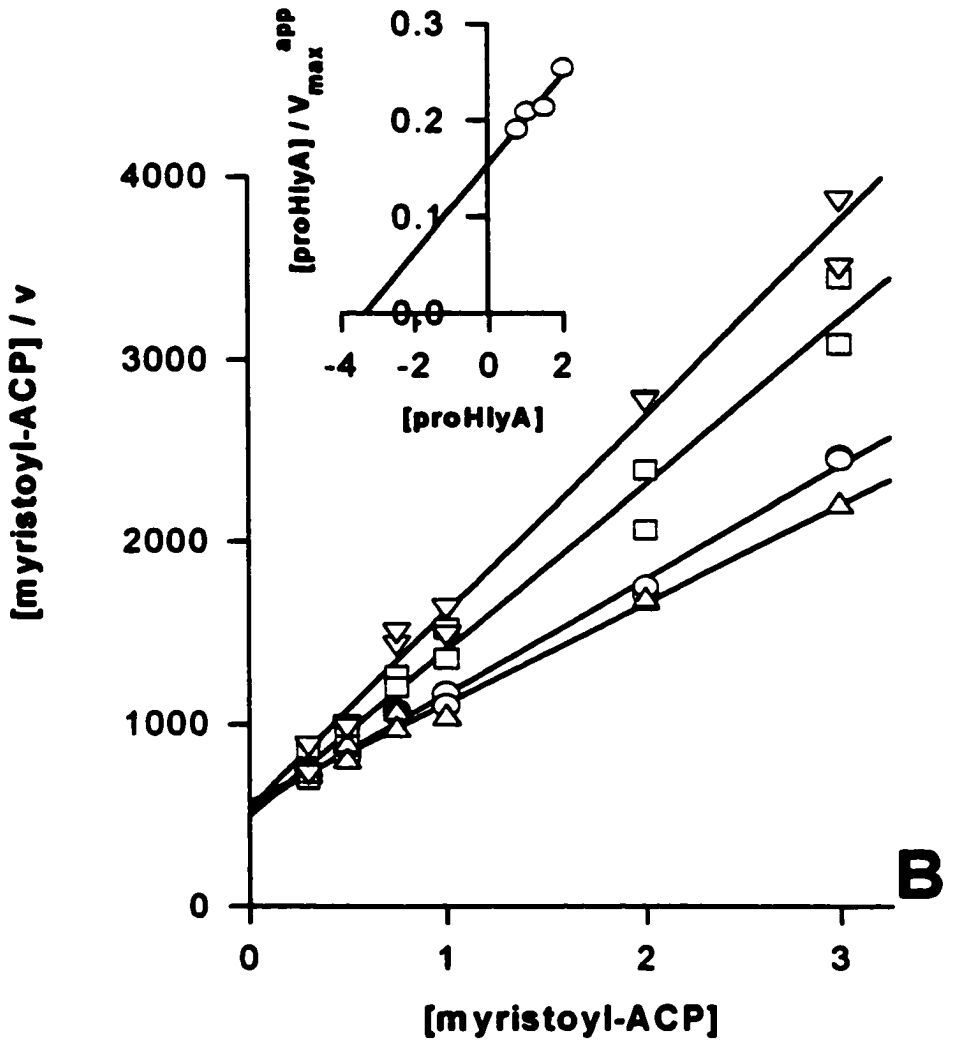


Figure 14 (continued): Myristoyl-ACP initial velocity data were obtained at the following different fixed concentrations of proHlyA: ∇, 0.34 μM; □ 1 μM; o, 1.5 μM; and Δ, 2 μM. Velocitys (v) were in identical arbitrary units. The inset shows the secondary plot graphical determination of the reaction kinetic parameters for proHlyA from the respective half-reciprocal plot slopes ($1/V_{max}^{app}$) at different [proHlyA] (μM). The linear correlation coefficient was 0.96.

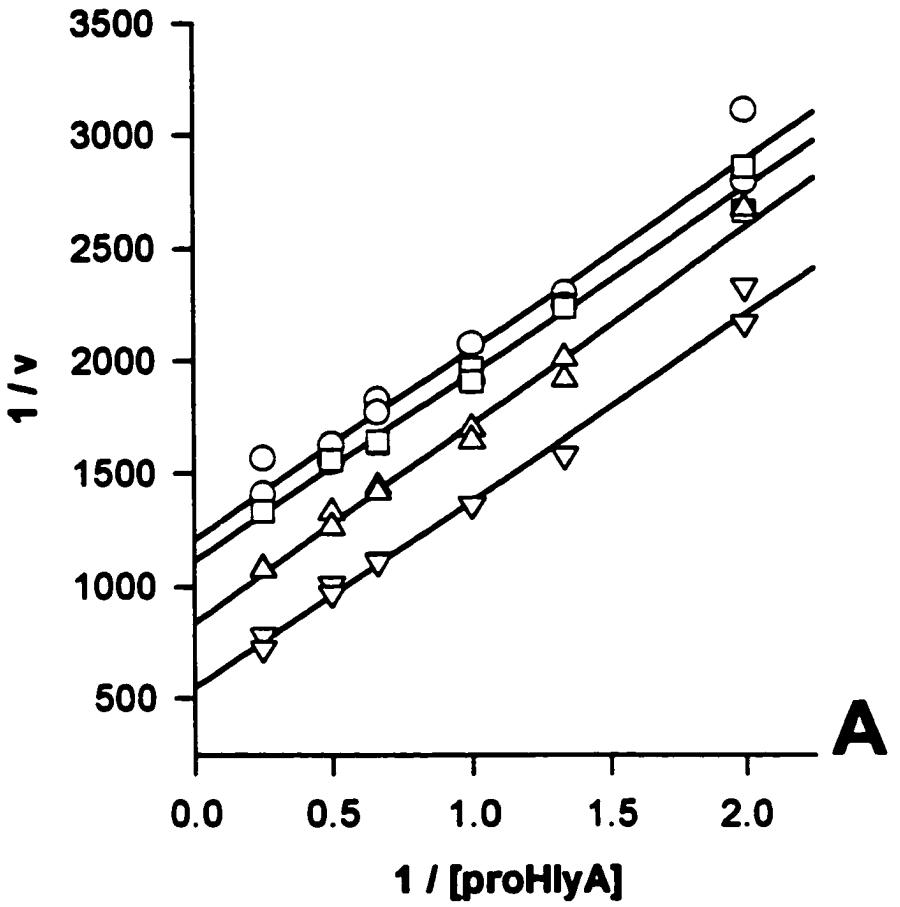


Figure 15: ProHlyA initial velocity data at different fixed non-saturating concentrations of myristoyl-ACP shown as double reciprocal plots (A) and as half-reciprocal plots (B).

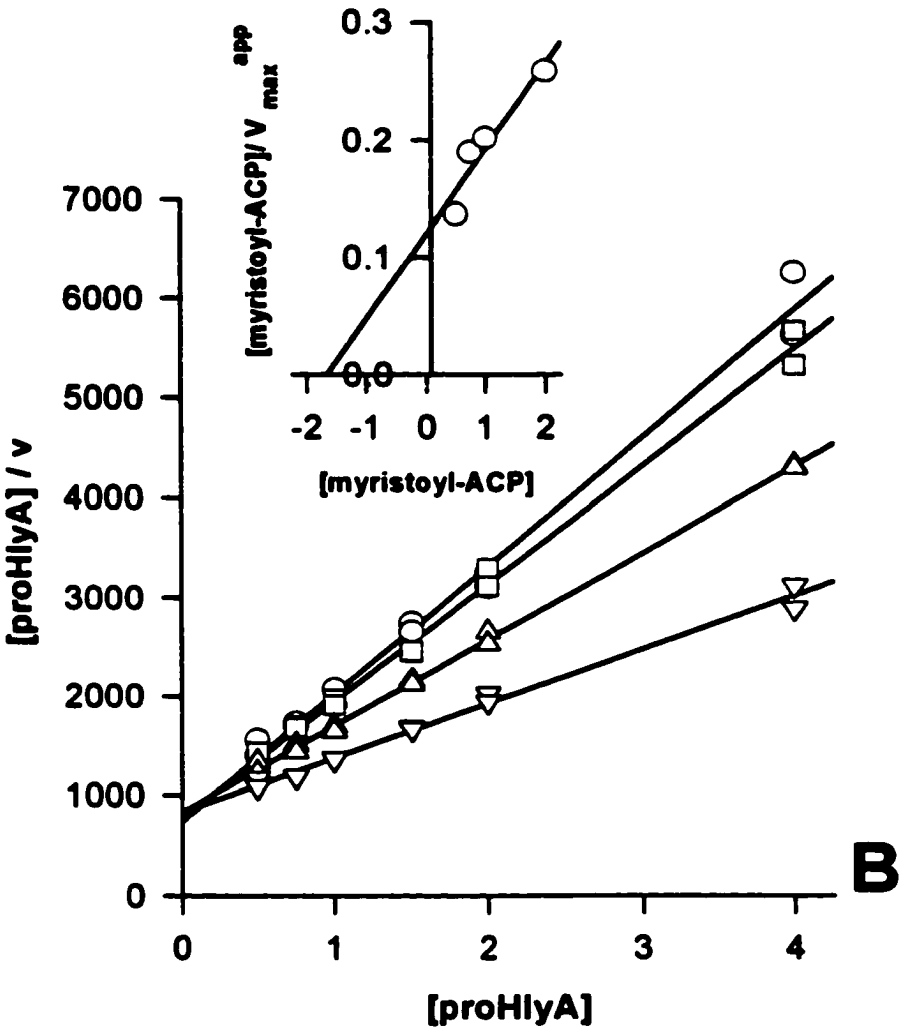


Figure 15 (continued): Initial velocity data for proHlyA were obtained at the following different fixed concentrations of myristoyl-ACP: o, 0.5 μM; □, 0.75 μM; Δ,1 μM; and ∇, 2 μM. Velocity (v) was in arbitrary units. The inset shows the secondary plot graphical determination of the reaction kinetic parameters for myristoyl-ACP from the respective half-reciprocal plot slopes ($1/V_{max}^{app}$) at different [myristoyl-ACP] (μM). The linear correlation coefficient was 0.96.

is shown to demonstrate that data describing parallel lines on a double reciprocal plot require convergence on the [S]/v axis of a half reciprocal plot (*83*). Throughout the remainder of this manuscript, kinetic data are displayed as half-reciprocal (Hanes) plots which has the best distribution of error throughout the plot (*84*, *85*). Each pattern shown by the two plotting methods was consistent with a bi bi substituted enzyme (bi bi ping pong) kinetic mechanism for HlyC. In contrast, a ternary complex mechanism would show a series of plots of differing intercepts on a half reciprocal plot. The kinetic constants of the reaction where both substrates are present in saturating amounts were determined from secondary plots shown in the insets of Figures 14B and 15B and also by inserting the kinetic parameters obtained from the primary plots (Figures 14 and 15) into rate equations (*73*). There was excellent agreement between the two methods. $K_m^{\text{myristoyl-ACP}}$ was 1.6±0.4 μM, and K_m^{proHlyA} was 3.6±1.2 μM; V_{max} was 14.0±1.8 pmoles of acyl group transferred/min. The correlation coefficient for the determination of these three values was 0.99 for each.

Product Inhibition Kinetics

HlyC acyltransferase activity was not effected by the addition of unlabelled palmitoyl-CoA or free fatty acid to the reaction. If, however, ACPSH or HlyA were added, the reaction was inhibited. These product inhibitions allowed further characterization of the enzyme mechanism. Initial velocity studies of a bi bi reaction can place it into two general mechanistic groups, ping pong or sequential, while product inhibition studies can provide a finer resolution of the kinetic mechanism. In order to

define further the kinetic mechanism of HlyC catalysis, the addition of different fixed concentrations of each of the products separately to initial reaction velocity studies for each substrate was done.

The addition of different fixed amounts of ACPSH to reactions containing variable amounts of proHlyA resulted in a family of linear plots, which appeared parallel on a double-reciprocal plot (not shown). The parallel lines were verified by plotting the data on a half reciprocal plot (Figure 16) where the intercepts converged on the [S]/v axis (*83*). The graphical patterns were consistent with uncompetitive inhibition where both V_{max}^{app} and K_m^{app} decreased in response to the presence of increasing fixed amounts of product, ACPSH. A similar pattern of inhibition was seen when different fixed amounts of ACPSH were added to reactions where myristoyl-ACP was the varied substrate (Figure 17). In both cases, K_m and V_{max} values changed by the same factor as [I] increased. Replots of $1/V_{max}^{app}$, the slopes of Figure 16 and 17, versus [I] were hyperbolic for ACPSH product inhibition with respect to both myristoyl-ACP and proHlyA (insets). These findings described a kind of mixed-type inhibition in which K_m and V_{max} change by the same factor resulting in parallel line double-reciprocal plots (not shown) (*86*). In contrast to linear uncompetitive inhibition where the curves continue to move apart as [I] increases, the displacement between the curves in Figures 16 and 17 became limiting as [I] increased (double-reciprocal plots not shown). This was shown by the hyperbolic replots (insets) and also by plots of initial reaction velocities against [I] at fixed [S] which showed a leveling of the velocities at higher [I] values rather than a continuous decrement of initial reaction velocities as [I] increased (data not shown).

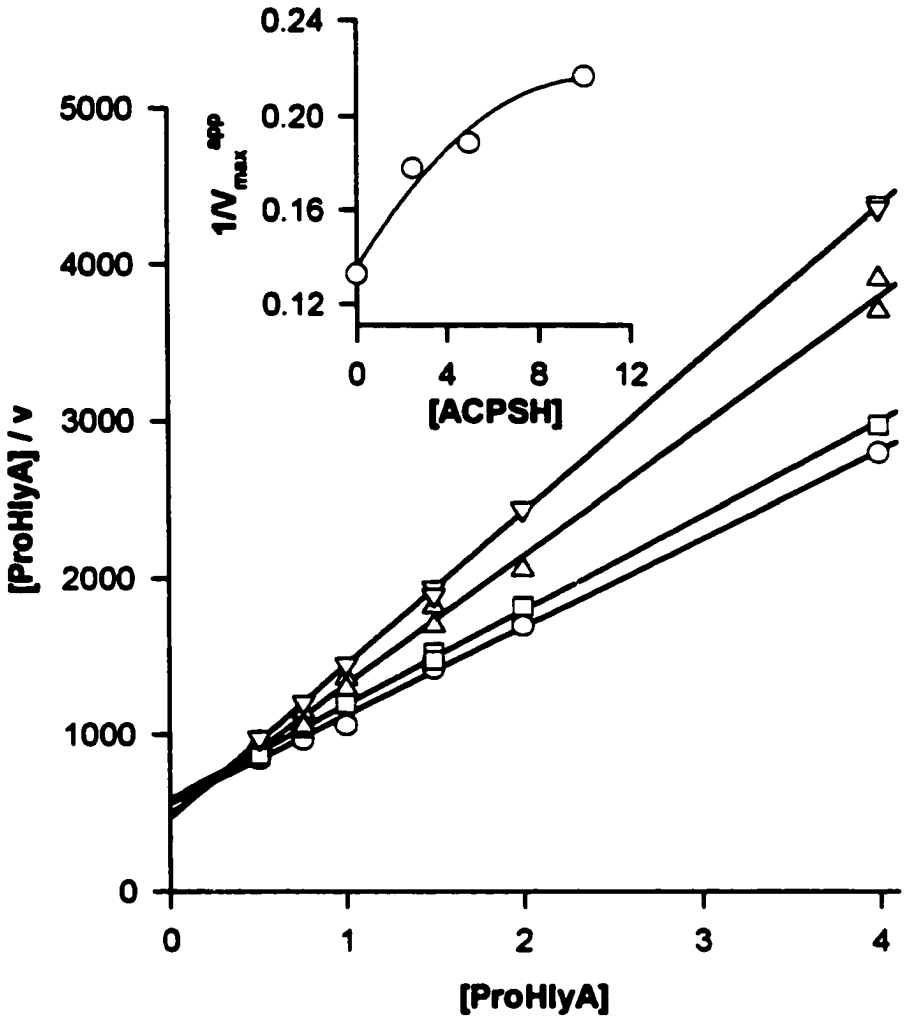


Figure 16: ACPSH inhibition with respect to proHlyA. Evaluation of the kinetic parameters of proHlyA at a fixed myristoyl-ACP concentration of 1 μM was done in the presence of the following concentrations of the product ACPSH: ∇, 10 μM; Δ, 5 μM; □, 2.5 μM; and o, 0 μM. Velocity (v) was in identical arbitrary units in all half-reciprocal plots. The inset shows the half-reciprocal plot slopes, $1/V_{max}^{app}$, versus the corresponding [ACPSH] (μM).

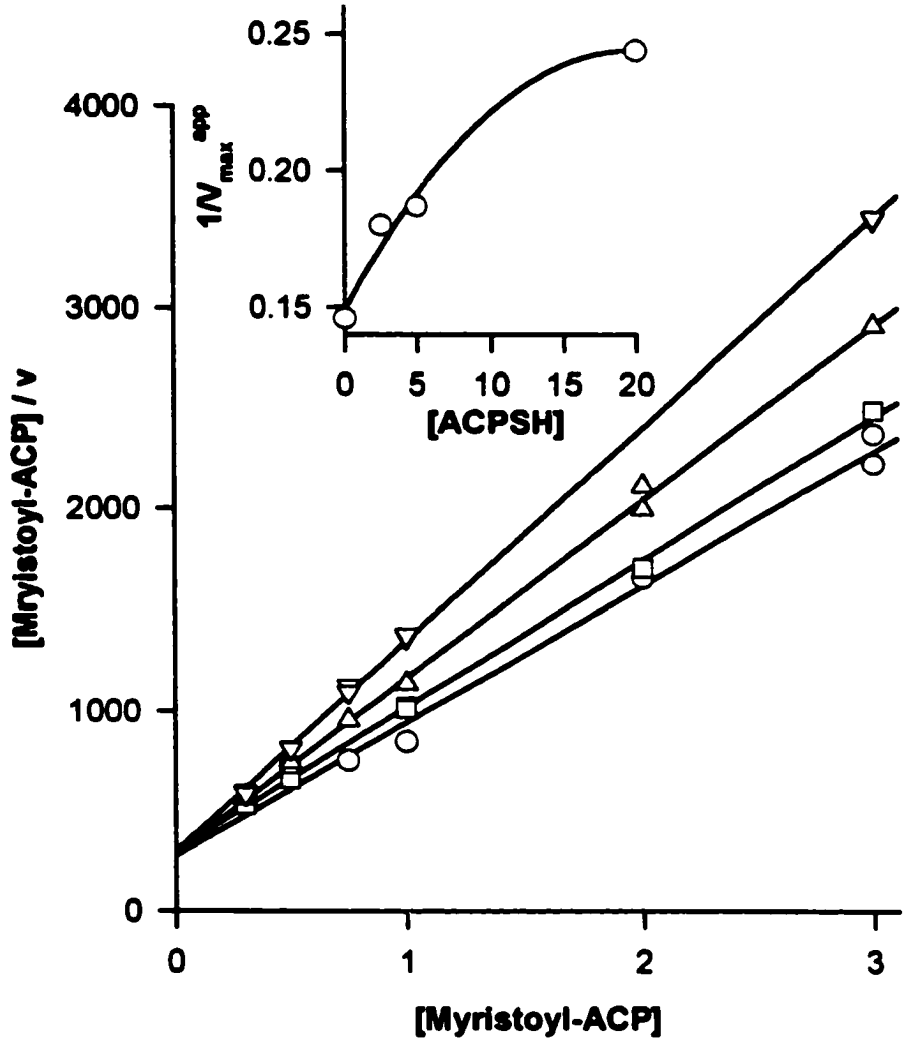


Figure 17: ACPSH inhibition with respect to myristoyl-ACP. Evaluation of the kinetic parameters of myristoyl-ACP was done at a fixed proHlyA concentration of 2 μM in the presence of the following concentrations of the product ACPSH: ∇, 20 μM; Δ, 5 μM; □, 2.5 μM; and o, 0 μM. Velocity (v) was in identical arbitrary units in all half-reciprocal plots. The inset shows the half-reciprocal plot slopes, $1/V_{max}^{app}$, versus the corresponding [ACPSH] (μM).

Thus ACPSH inhibition with respect to both myristoyl-ACP and proHlyA at fixed, unsaturating values of each other was mixed-type inhibition.

Prior to studying the effects of the product, HlyA, on reaction rates, any potential for additional acylation of HlyA was examined. Purified HlyA produced *in vivo* was obtained from *E. coli* containing pEK50, a plasmid encoding for the hemolysin operon. The *in vivo* produced toxin was subjected to possible acylation in the assay milieu, which contained radiolabeled myristoyl-ACP and HlyC. HlyA so treated was not acylated. It was not visualized on fluorography after extended exposure nor was it radioactive when isolated and measured in a liquid scintillation counter.

Upon addition of different fixed amounts of the product HlyA to reactions containing variable amounts of myristoyl-ACP, the resulting primary double-reciprocal plots all intersected at the same point on the 1/v axis (not shown). In confirmation of this pattern, the half-reciprocal primary plots (Figure 18) showed a series of parallel lines. These findings were diagnostic of competitive inhibition. The primary plots will not distinguish among the following types of competitive inhibition: one-site, pure competitive inhibition; two-site, pure competitive inhibition, and partial competitive inhibition (*87*). The three types of competitive inhibition can be distinguished by plotting reaction velocity versus [I] at a fixed substrate concentration. In partial competitive inhibition, the resulting curve reaches a plateau at a limiting velocity, and the reaction rate shows no tendency to approach zero. In contrast, both one-site and two-site types of pure competitive inhibition will give plots that indicate that reaction velocity approaches

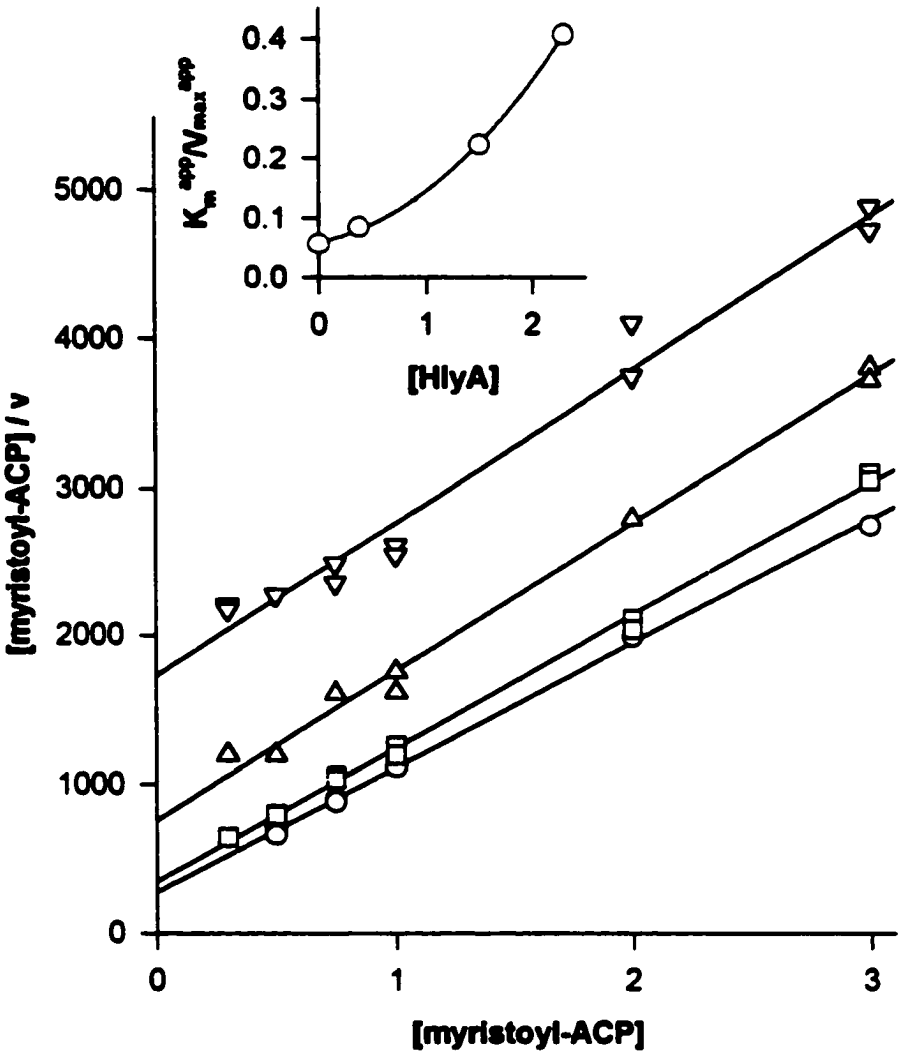


Figure 18: HlyA inhibition with respect to myristoyl-ACP. Evaluation of the kinetic parameters of myristoyl-ACP was done at a fixed proHlyA concentration of 2 μM in the presence of the following concentrations of the product HlyA: ∇, 2.3 μM; Δ, 1.5 μM; □, 0.3 μM; and o, 0 μM. Velocity (v) was in identical arbitrary units in all half-reciprocal plots. The inset shows the double reciprocal plot slopes, K_m^{app}/V_{max}^{app}, versus the corresponding [HlyA] (μM).

zero upon sufficiently increasing [I]. Such a plot of the data from Figure 18 showed no leveling off of the reaction velocity as [I] increased so the inhibition was not partial competitive inhibition (Figure 20A). Furthermore, as shown in the inset in Figure 18, the replot of double reciprocal plot slopes versus [I] was parabolic; this is diagnostic of two site, competitive inhibition (*87*). Similar plots of data describing one-site pure competitive or partial competitive systems would, in comparison, yield linear and hyperbolic replots, respectively. Dixon plots (reciprocal velocity values at fixed [S] against [I]) and plots of K_m^{app} versus [I] were both parabolic (not shown) as would be expected in two-site, pure competitive inhibition (*87*).

When different fixed amounts of the product HlyA were added to reactions where proHlyA concentration was varied, a primary plot pattern like that described above for HlyA inhibition with respect to myristoyl-ACP was observed. HlyA inhibited with respect to proHlyA as shown in Figure 19 where the primary half-reciprocal plots at different HlyA concentrations formed parallel lines. The corresponding double-reciprocal plots showed increasing slopes with increasing inhibitor concentration while all plots intersected on the 1/v axis (not shown). These patterns indicated competitive inhibition. As mentioned above, there are several types of competitive inhibition, and all have the same primary plot pattern. A secondary plot of initial velocity versus [HlyA] at fixed [S] showed a tendency for the velocity to plateau at higher concentrations of HlyA as shown in Figure 20B. This was in contrast to the results from above shown in Figure 20A where a continuously decreasing velocity was observed as inhibitor concentration increased. This finding indicated partial competitive inhibition. In confirmation of

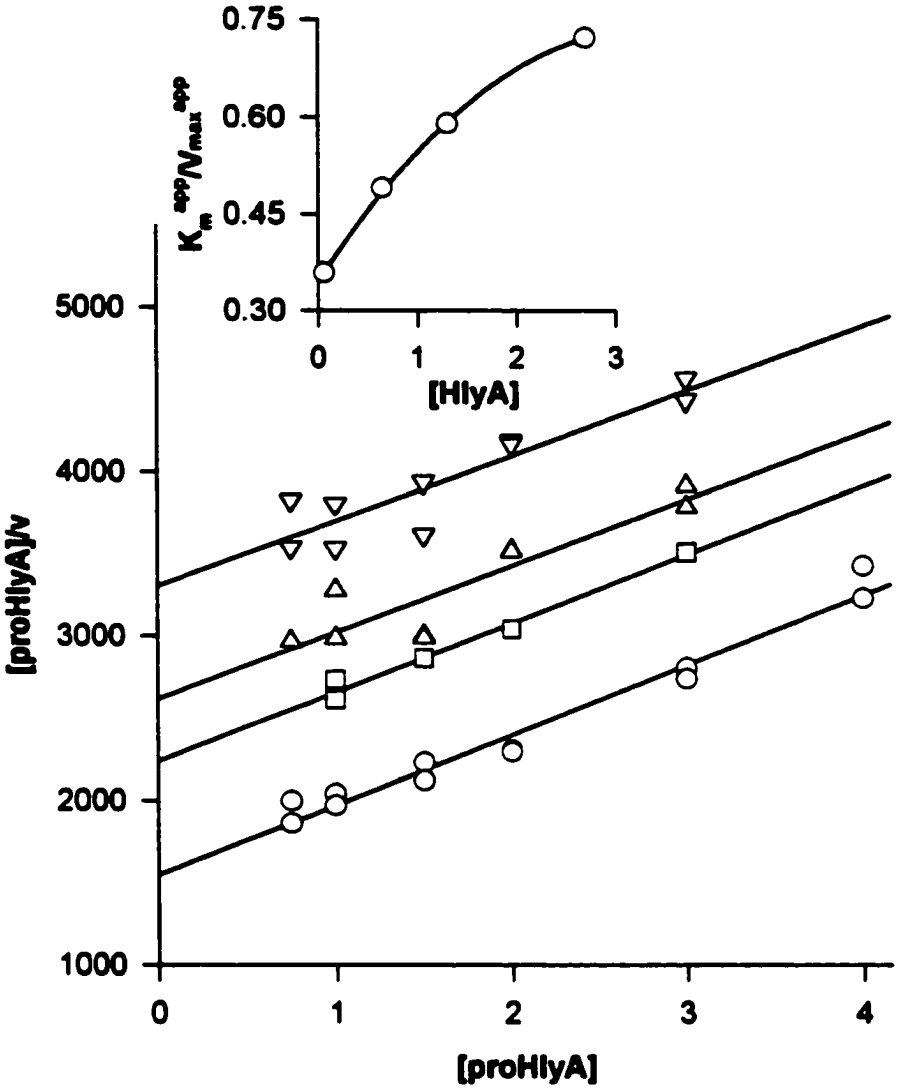


Figure 19: HlyA inhibition with respect to proHlyA. Evaluation of the kinetic parameters of proHlyA was done at a fixed myristoyl-ACP concentration of 1 μM in the presence of the following concentrations of the product HlyA: ∇, 2.7 μM; Δ, 1.3 μM; □, 0.6; and o, 0.065 μM. Velocity (v) was in identical arbitrary units in all half-reciprocal plots. The inset shows the double reciprocal plot slopes, K_m^{app}/V_{max}^{app}, versus the corresponding [HlyA] (μM).

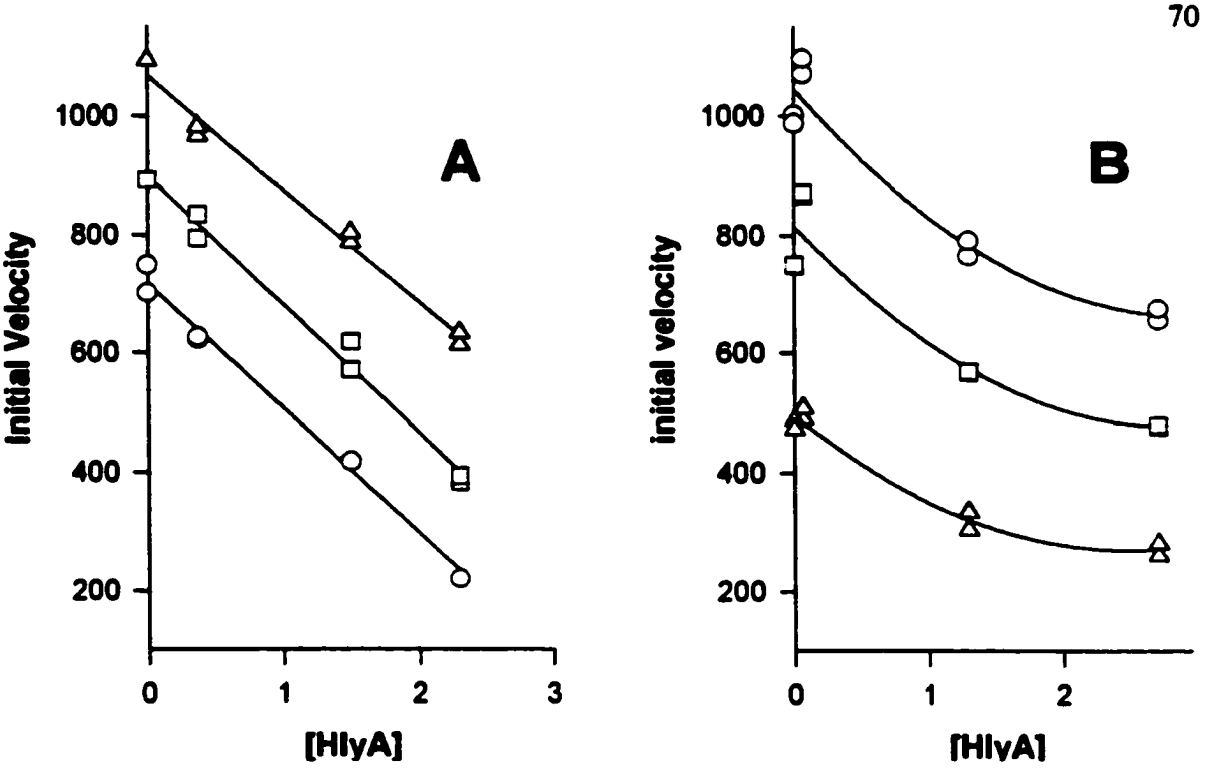


Figure 20: Plots of initial velocities at fixed [S] in the presence of product, [HlyA] (μM) with myristoyl-ACP (A) or proHlyA (B) as the variable substrates. Initial reaction velocities are given in dpm. In (A), in the presence of different concentrations of HlyA (μM), reaction velocities were determined at the following different [myristoyl-ACP]: Δ, 3 μM; □, 1 μM; and o, 0.5 μM. [ProHlyA] was fixed at 2 μM. In (B), in the presence of different concentrations of HlyA (μM), reaction velocities were determined at the following different [proHlyA]: o, 3 μM; □, 2 μM; and Δ, 1 μM. [Myristoyl-ACP] was fixed at 1 μM.

partial competitive inhibition (also called hyperbolic inhibition), the replot of double reciprocal plot slopes versus [HlyA] was hyperbolic (Figure 19 inset) (*88*). Unlike the situation with simple linear inhibition, K_i cannot be obtained from the intercept of the replot, but the equation for partial competitive inhibition can be converted into a linear relationship (*89*). In this way, a K_i of 4.3 μM HlyA was obtained from a linear plot (Figure 21) of $1/\Delta$ slope against 1/[HlyA].

The partial competitive inhibition seen when [HlyA] was varied with respect to proHlyA may be regarded as a variant of mixed-type inhibition (*90*); the basis for this will be discussed later. The product inhibitions for the HlyC catalyzed reaction are summarized in Table 7. At fixed, nonsaturating concentrations of proHlyA or myristoyl-ACP respectively, ACPSH inhibitions with respect to myristoyl-ACP or proHlyA were each mixed-type inhibition. HlyA inhibition with respect to myristoyl-ACP, at fixed, nonsaturating concentrations of proHlyA, gave a pattern of competitive inhibition. A different pattern was obtained for HlyA inhibition with respect to proHlyA at fixed nonsaturating concentration of myristoyl-ACP; a pattern consistent with a form of mixed-type inhibition was observed. The product inhibition patterns of the acyltransferase reaction shown in Table 6 conformed to an uni uni iso uni uni ping pong kinetic mechanism with isomerization of the F form of the enzyme (*91*).

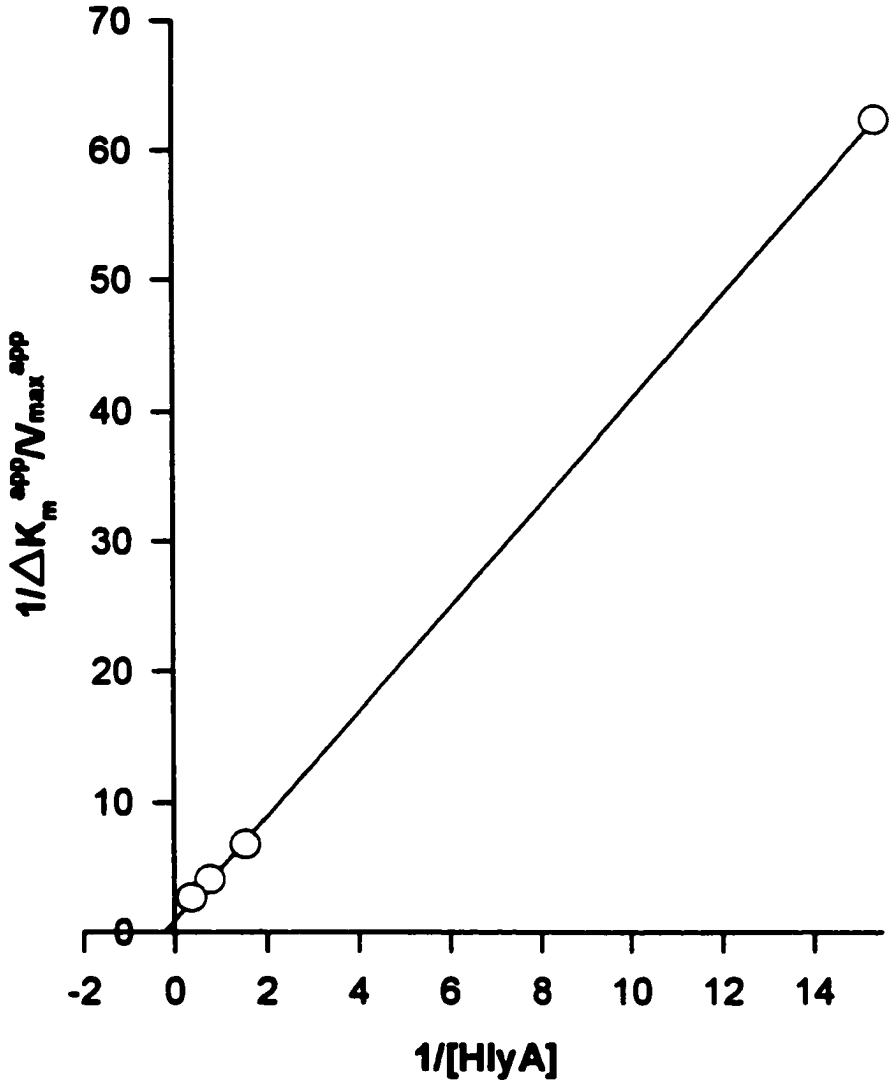


Figure 21: Determination of the K_i for HlyA inhibition with respect to proHlyA at fixed [myristoyl-ACP]. The relationship between K_m^{app}/V_{max}^{app} (double-reciprocal plot slopes or half-reciprocal plot intercepts) and different [HlyA] provides $-K_i$ on the x intercept when the relationship is linear (*89*). Data are from Fig. 19. The linear correlation coefficient was 1.

Table 6: PRODUCT INHIBITION PATTERNS[a]

variable substrate	product	fixed substrate[b]	inhibition pattern[c]
myristoyl-ACP	ACPSH	proHlyA	mixed-type
proHlyA	ACPSH	myristoyl-ACP	mixed-type
myristoyl-ACP	HlyA	proHlyA	competitive
proHlyA	HlyA	myristoyl-ACP	mixed-type

[a]The assay procedure and reaction conditions are given in Experimental Procedures. [b]Nonsaturating concentrations of fixed substrates were used. [c]Inhibition pattern notations are those described by Segel (*91*).

Chemical Mechanism of HlyC

Chemical Modification of HlyC

As discussed earlier, HlyC appears to be a unique acyltransferase resembling no other known acyltransferases. However, it has been demonstrated that some RTX C proteins can acylate pro-RTX toxins from different organisms, indicating a similar mode of action for the various RTX C acyltransferases. To elucidate the chemical mechanism of this unique enzyme, the deduced amino acid sequences of 13 different RTX C proteins were compared. The sequence alignment shown in Figure 22 reveals a high degree of identity, with the majority of homology concentrated in the N-terminal region. Possibly, the N-terminal region of HlyC may be involved in the acyl transfer reaction; whereas, the unique C-terminal regions of the various acyltransferases may be involved in recognition of the diverse protoxins. Out of 170 residues in HlyC, 37 are totally conserved in the 13 RTX C gene products (Figure 22). Of particular interest to us were those residues that could covalently bear an acyl group since HlyC forms an acyl-enzyme intermediate in the presence of its first bound substrate, acyl-ACP. To investigate the chemical mechanism for the acyl transfer reaction, HlyC was covalently modified by a number of chemical reagents. The reactions for the various chemical modification reagents used in this study are shown in Figure 23.

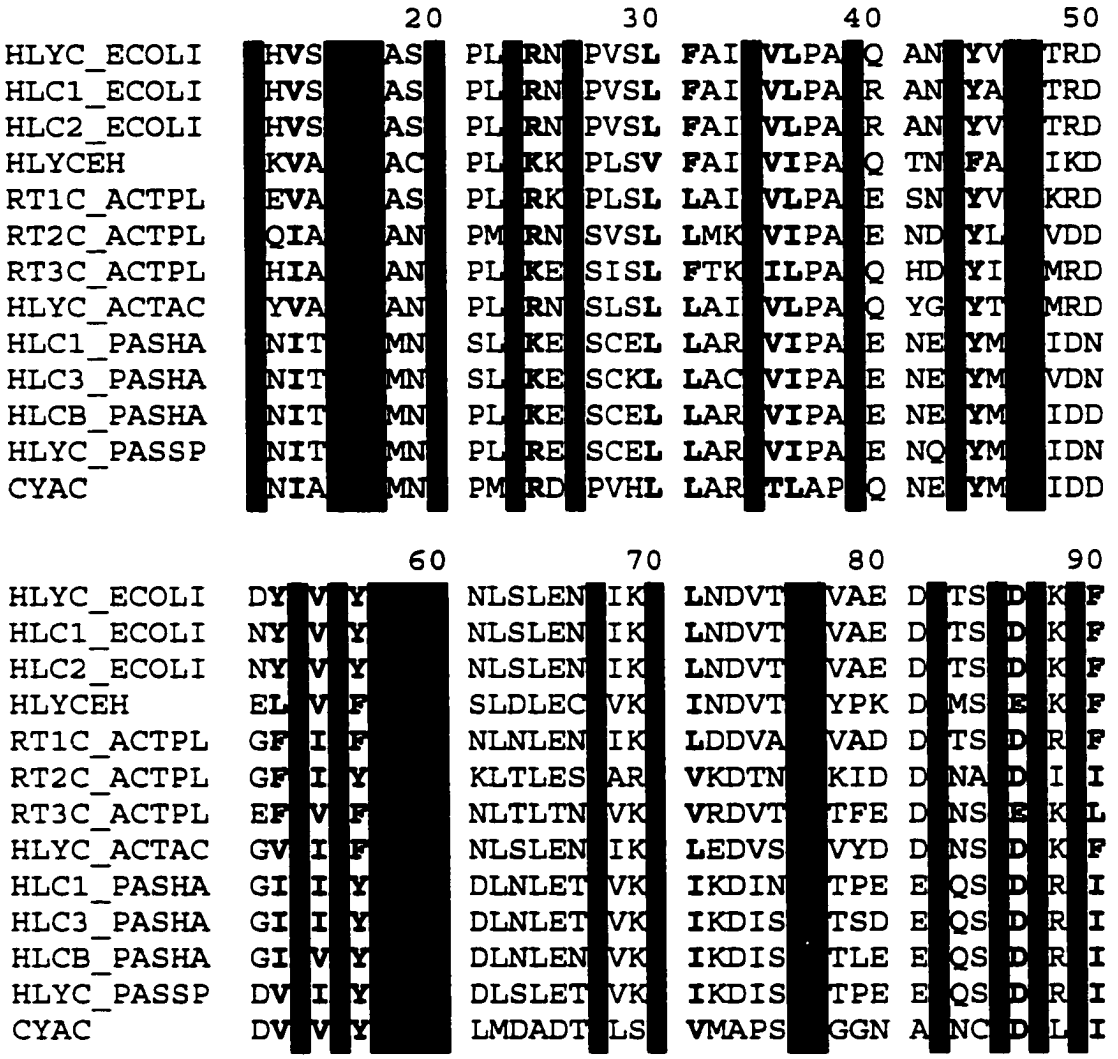


Figure 22: Partial amino acid sequence alignment of 13 RTX toxin C gene products. HLYC_ECOLI, *E. coli* HlyC, pHly152 encoded, acession no. p06736; HLC1_ECOLI, *E. coli* strain J96 HlyC, chromosome encoded, accession no. p09984; HLC2_ECOLI, *E. coli* strain 2001 HlyC, chromosome encoded, accesion no. p09985; HLYCEH, *E. coli*

Figure 22 (continued): 0157:H7 strain EDL 933 HlyC, plasmid encoded, translation of nucleotide sequence accesion no. x80891; RT1C_ACTPL, *A. pleuropneumoniae* Apx-IC, accesion no. p55132; RT2C_ACTPL, *A. pleuropneumoniae* Apx-IIC, accesion no. p15376; RT3C_ACTPL, *A. pleuropneumoniae*, Apx-IIIC, accession no. q04474; HLYC_ACTAC, *A. actinomycetemcomitans* LktC, accession no. p16461;

Figure 22 (continued): HLC1_PASHA, *P. haemolytica* serotype A1 LktC, accession no. p16533; HLC3_PASHA, *P. haemolytica* sertoype T3 LktC, accession no. p55120; HLCB_PASHA, *P. haemolytica* serotype A11 LktC, accession no. p55121; HLYC_PASSP, *P. haemolytica*-like sp. LktC, accession no. p55124; CYAC, *B. pertussis* CyaC, translation of nucleotide sequence accession no. m57286. Nonconserved amino acids are shown in uppercase letters; conserved residues are shown in bold uppercase letters. Amino acid residues that are identical in all 13 RTX C proteins are shaded. The sequence aligment was done using the Hibio Prosis protein analysis software by Hitachi. Amino acid sequence number refers to positions in the *E. coli* HlyC encoded by plasmid pHly152.

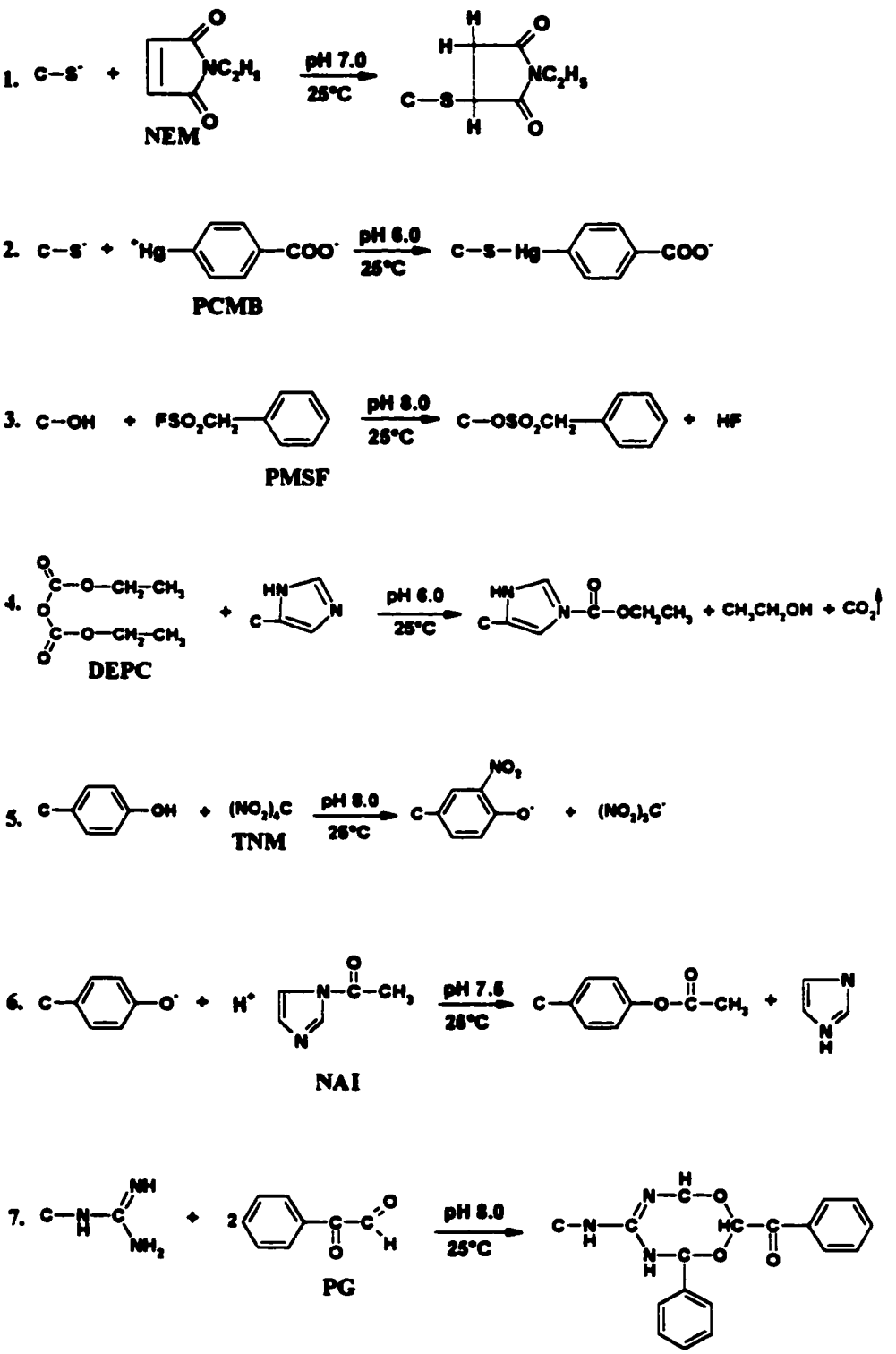


Figure 23: Chemical reactions for different chemical modifiers with their target residues (77).

The RTX acyltransferases all contain a single conserved Cys residue, Cys 58 in HlyC. NEM, which reacts specifically with sulfhydryl groups (*78*), did not affect enzyme activity (Table 7). Since the nature of the environment surrounding the reactive group can effect its reactivity with various chemical reagents, a second more hydrophobic sulfhydryl-specific modifying reagent, PCMB (*92*), was also tested as an inhibitor for the acyltransferase. Again, there was no decrease in enzyme activity (Table 7), suggesting a –SH group is not necessary for catalysis. HlyC was also insensitive to PMSF or AEBSF (Table 7), which could potentially modify one or more of the three conserved serines; residues 20, 58, and 76, suggesting that the enzyme is not a serine-dependent acyltransferase.

HlyC contains one conserved His at position 23, which could be involved in the enzymatic transfer of an acyl chain. His could function as a base deprotonating a nucleophilic residue accepting the acyl chain or possibly serve as the acyl acceptor, although an acyl-imidazole enzyme intermediate has never been reported previously. HlyC was rapidly inactivated by DEPC at pH 6.0 (Table 7 and Figure 24). At this pH, the reagent is highly specific for histidine residues producing N-carbethoxyhistidine (Figure 23 reaction 4), while at pH 8.0 the reagent may modify cysteine, tyrosine, and lysine residues (*79*). HlyC treated with 1 mM DEPC at pH 6.0 retained 16±1% acyltransferase activity as compared to 68±0% remaining activity at pH 8.0 suggesting a specificity for histidine (Table 7).

Table 7: INHIBITION OF HLYC ACYLTRANSFERASE ACTIVITY BY CHEMICAL MODIFIERS

Modifier	[Modifier] (mM)	Reaction Conditions[a]	% Activity Remaining[b]
NEM	1.0	0.1 M KPO_4 (pH 7.0), 15 min	100±10
PCMB	1.0	20 mM Mes (pH 6.0), 10 min	117±1
PMSF	1.0	25 mM Hepes (pH 8.0), 10 min	95±10
AEBSF	1.0	25 mM Hepes (pH 8.0), 10 min	126±0
DEPC	1.0	0.1 M KPO_4 (pH 6.0), 10 min	16±1
DEPC	1.0	0.1 M KPO_4 (pH 8.0), 10 min	68±0
TNM	1.5	0.05 M Tris (pH 6.0), 30 min	81±1
TNM	1.5	0.05 M Tris (pH 8.0), 30 min	20±1
NAI	1.3	50 mM Hepes (pH 7.5), 30 min	83±2
PG	5.0	25 mM Hepes (pH 8.0), 1 h	10±2

[a]All chemical modification reactions were performed at 25 °C. [b]Activity is the % of the control reaction without modifier. As described in the Materials and Methods a separate control was completed for each modification reaction. On average, control reactions containing 10 μg of HlyC gave 35.0±2.5 pmoles of acyl group transferred/assay.

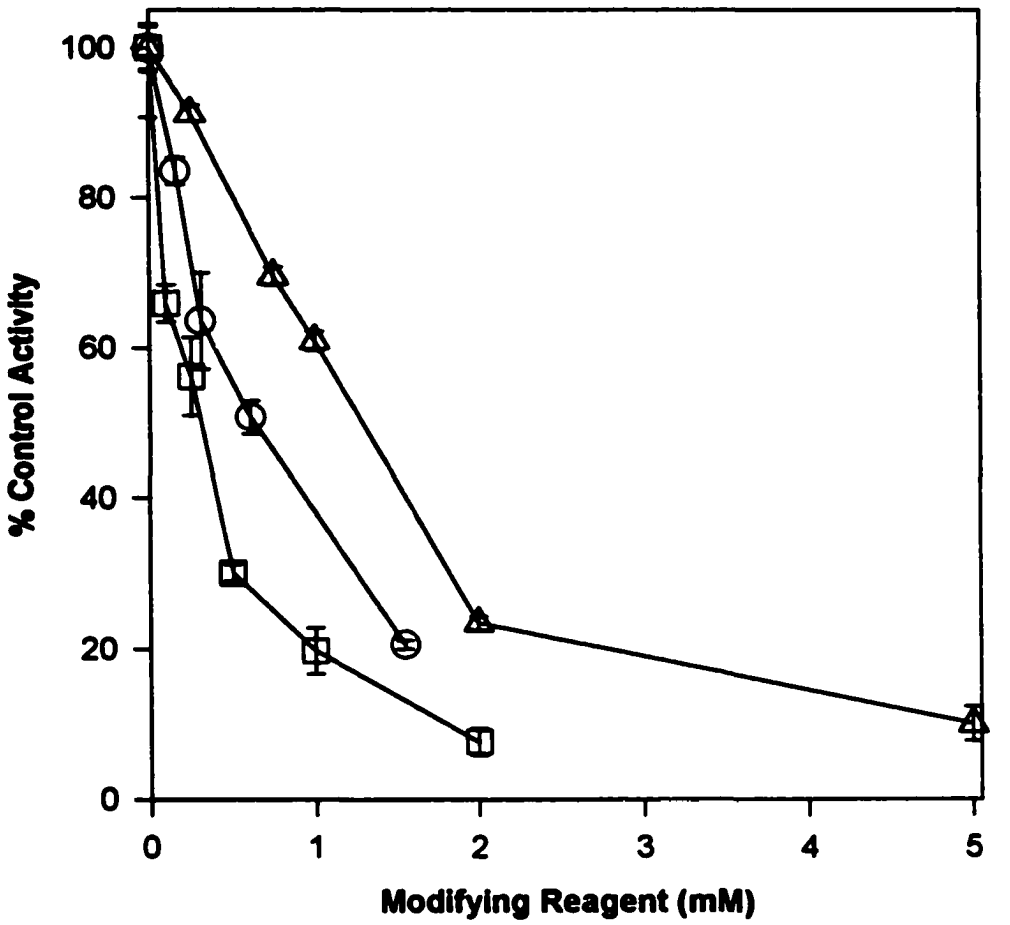


Figure 24: Inhibition of HlyC acyltransferase activity by protein modification reagents. HlyC was incubated with increasing concentration of (□) DEPC, (o) TNM, or (Δ) PG. Chemical modification reactions were performed as described in the Materials and Methods with each reaction having its own control not exposed to the chemical modifier. Control activities for the DEPC, TNM, and PG modification experiments were 29.0±3.0, 40.0±1.0, and 37.0±1.0 pmoles acyl group transferred/assay, respectively. Assays contained 10 μg HlyC.

Treatment of DEPC modified HlyC with 100 mM NH_2OH (pH 7.0) (Figure 25) resulted in a time dependent re-activation of the enzyme activity with 87±1% acyltransferase activity after 4 h, indicating that inactivation was not due to lysine, tyrosine, or sulfhydryl modification (*79*). DEPC modification of cysteine or lysine residues is not reversed by NH_2OH treatment and modified tyrosines are reversed at a much slower rate (*79*). Incomplete reactivation of the enyzme could be attributed to either modification of lysyl residues which are not affected by hydroxylamine treatment; but are essential for HlyC function, or possibly due to the formation of a disubstituted histidyl derivative that is not regenerated by hydroxylamine (*79*). Modification of hisitidine by DEPC was confirmed by difference spectroscopy of DEPC modified and native HlyC, showing an absorption maximum of 242 nm characteristic for N-carbethoxyhistidine with no significant decrease in abosrbance at 278 nm characteristic of ethoxyformylation of tyrosine (Figure 26) (*79*). The inactivation of the acyltransferase by DEPC followed pseudo first-order kinetics and correlated with the formation of N-carbeothoxyhistidine as shown by increasing absorbance at 242 nm over time (Figure 27).

HlyC contains two conserved tyrosines, Tyr 70 and Tyr 150, that could serve as acyl acceptors forming oxygen esters. At pH 8.0, TNM can specifically modify tyrosine residues producing 3-nitrotyrosine (Figure 23 reaction 5); whereas, at a pH 6.0 the reagent oxidizes sulfhydryl groups (*93*, *94*, *95*). HlyC was inactivated by TNM at pH 8.0 (Table 7 and Figure 24) with only 20±1% activity remaining at pH 8.0 as compared to

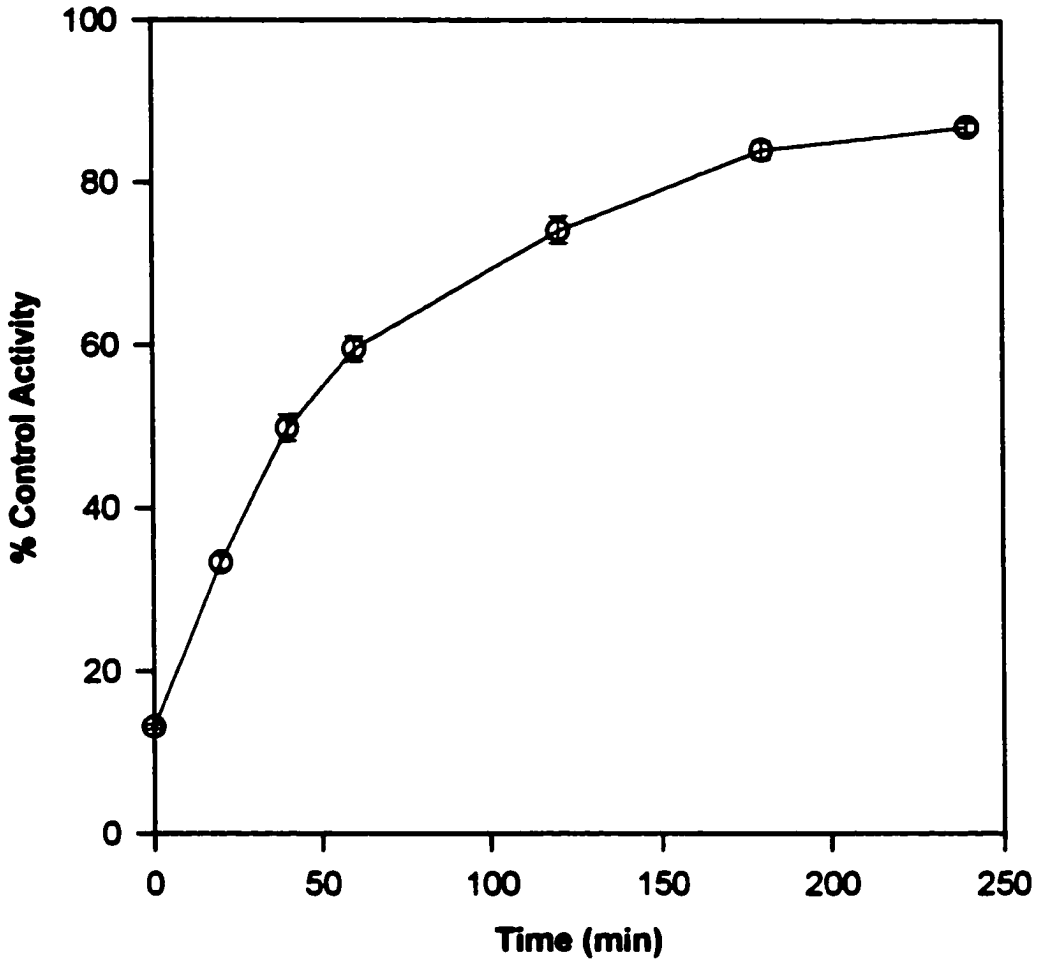


Figure 25: Reversibility of DEPC modification of HlyC. HlyC was incubated with 2 mM DEPC for 10 min at 25 °C until 13% acyltransferase activity remained followed by addition of NH_2OH (pH 7.0) to a concentration of 100 mM. At various time points, aliquots containing 10 μg of HlyC were removed and acyltransferase activity measured. Activity was expressed as the percentage of a control reaction not exposed to DEPC but incubated with 100 mM NH_2OH (pH 7.0) for 4 hrs. Control HlyC (10 μg) activity was 50.0±1.0 pmoles acyl group transferred/assay.

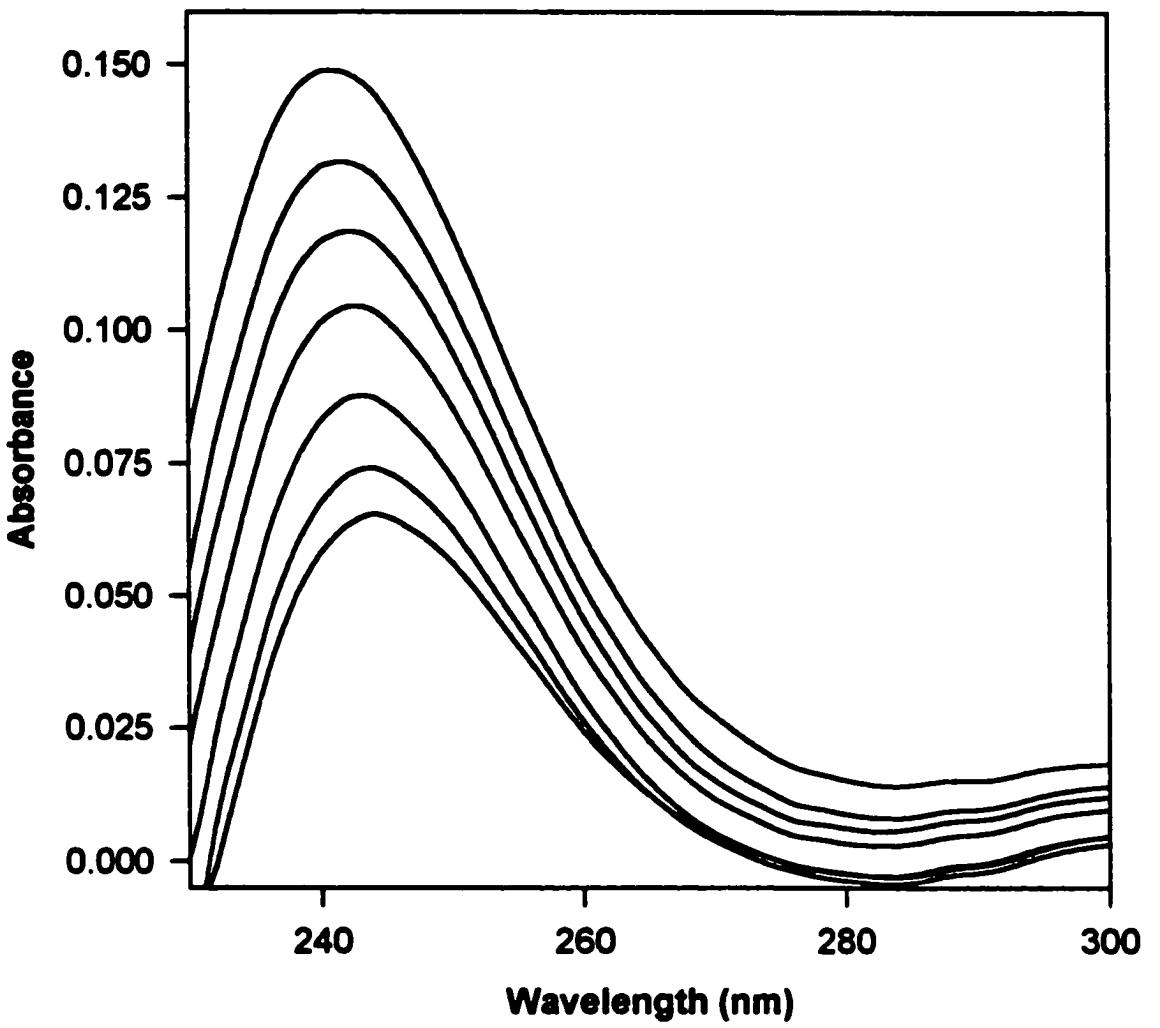


Figure 26: UV absorbance difference spectra demonstrating the effect of DEPC modification of HlyC. A 1.0 mg/mL S-tag-HlyC solution in a double sectored cuvette was modified with 1 mM DEPC in 0.1 M KPO_4 (pH 6.0) at 25 °C as described in Materials and Methods. Spectra were recorded at 1, 2, 4, 6, 10, 15, and 20 min intervals. The difference spectrum exhibits an absorbance peak at 242 nm that increases over time.

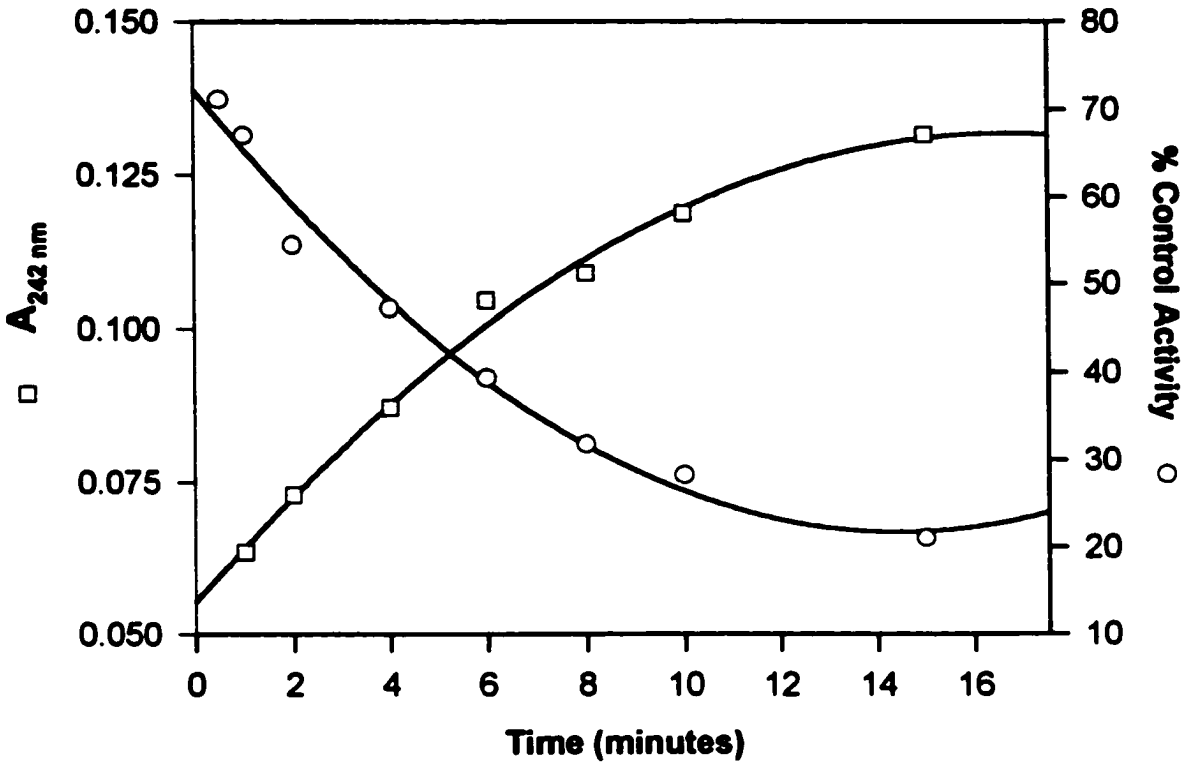


Figure 27: Correlation between the rate of DEPC modification (□) and loss of HlyC acyltransferase actvity (o). S-tag-HlyC in 0.1 M KPO_4 (pH 6.0) was treated with 1 mM DEPC at 25 °C and at various time intervals 10 μg aliquots of HlyC were removed and assayed for activity (o). Activity is expressed as the percentage of a control reaction (38.0±2.0 pmoles acyl group transferred/assay) containing 10 μg HlyC that was not exposed to DEPC. For absorbance studies, S-tag-HlyC modified under the same conditions was monitored for change in the absorbance at 242 nm.

81±1% activity remaining at pH 6.0 indicating that inactivation by TNM is not due to thiol oxidation. Modification of tryptophan residues was not considered likely since this reaction takes place at pH 9.0 or greater (*96*, *97*). Comparison of the modified and unmodified HlyC spectra showed an absoprtion maximum at 428 nm (Figure 28), consistent with the formation of nitrated tyrosine (*94*). TNM inactivation followed pseudo first-order kinetics and this decrease correlated with the formation of 3-nitrotyrosine as shown by the increase in absorbance at 428 nm over time (Figure 29).

TNM may induce the precipitation of some proteins through protein crosslinking resulting in loss of enzyme activity that is unrelated to tyrosine nitration (*98*, *99*). Possible cross linking of HlyC was tested by treating the enzyme with TNM followed by SDS-PAGE and western blotting using the S-protein alkaline phosphatase conjugate. There was no evidence of dimer, trimer, or large aggregate formation indicating that inactivation of HlyC was not due to protein cross linking (data not shown).

Like TNM, N-acetylimidazole under mild conditions reacts specifically with tyrosyl groups of proteins (*100*). Unlike TNM, the reaction directly effects the –OH group of the residue forming O-acetyltyrosine (Figure 23 reaction 6). HlyC activity was not affected by modification with N-acetylimidazole; 83±2% of acyltransferase activity remained after treatment with 1.3 mM of the reagent (Table 7). Acetylation of tyrosyl residues within HlyC was confirmed by a decrease in the absorbance at 278 nm (data not shown). The small decrease in activity probably stems from modification of lysyl residues (*100*). Acetylation of tyrosine residues had no effect on acyltransferase activity but, nitration of tyrosines almost completely inactivated the enzyme, suggesting that a

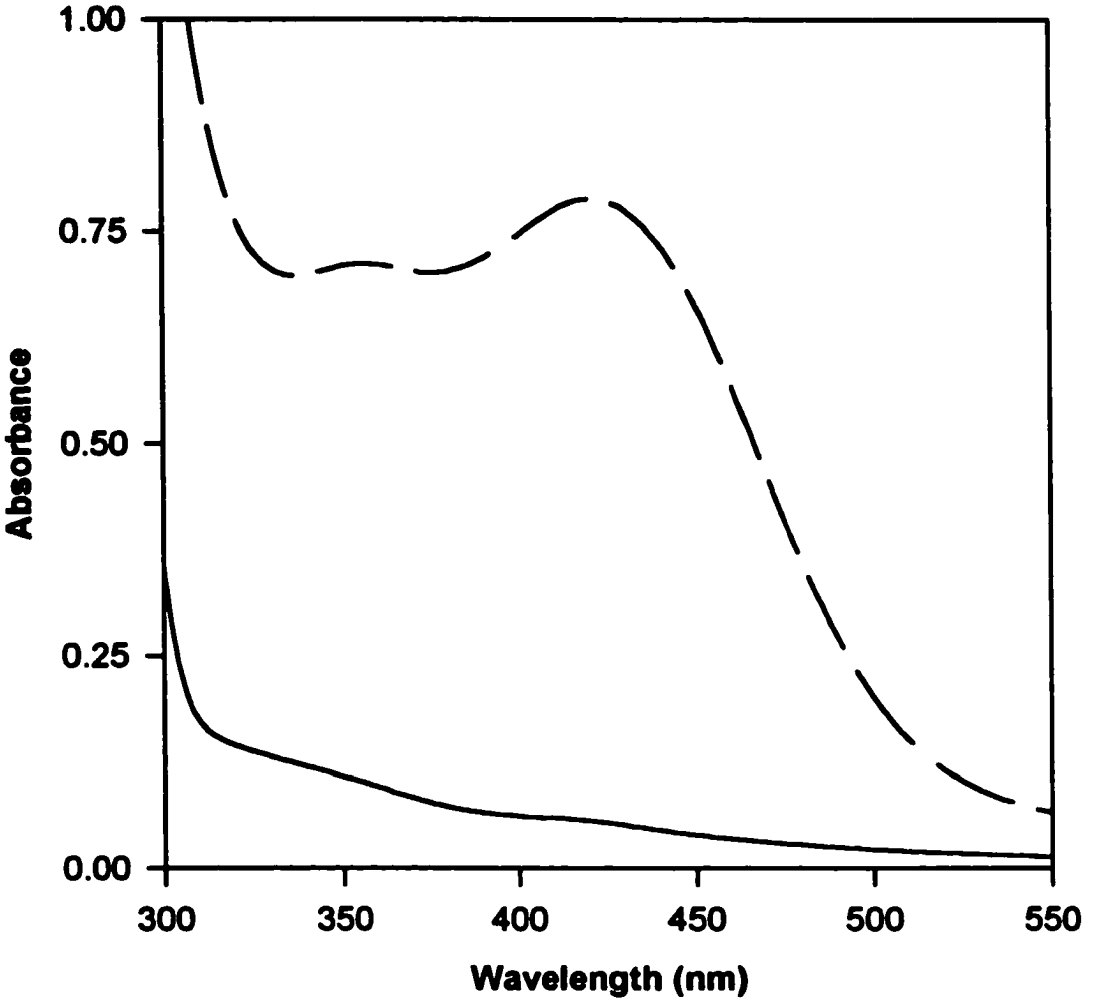


Figure 28: UV-Vis Spectra of TNM modified (- - -) and unmodified (—) HlyC. An 2.72 mg/mL S-tag-HlyC solution in 0.05 M Tris pH 8.0 was modified with a 50-fold molar excess of TNM (6.0 mM) for 30 minutes at 25 °C in a double sectored cuvette as described in Materials and Methods.

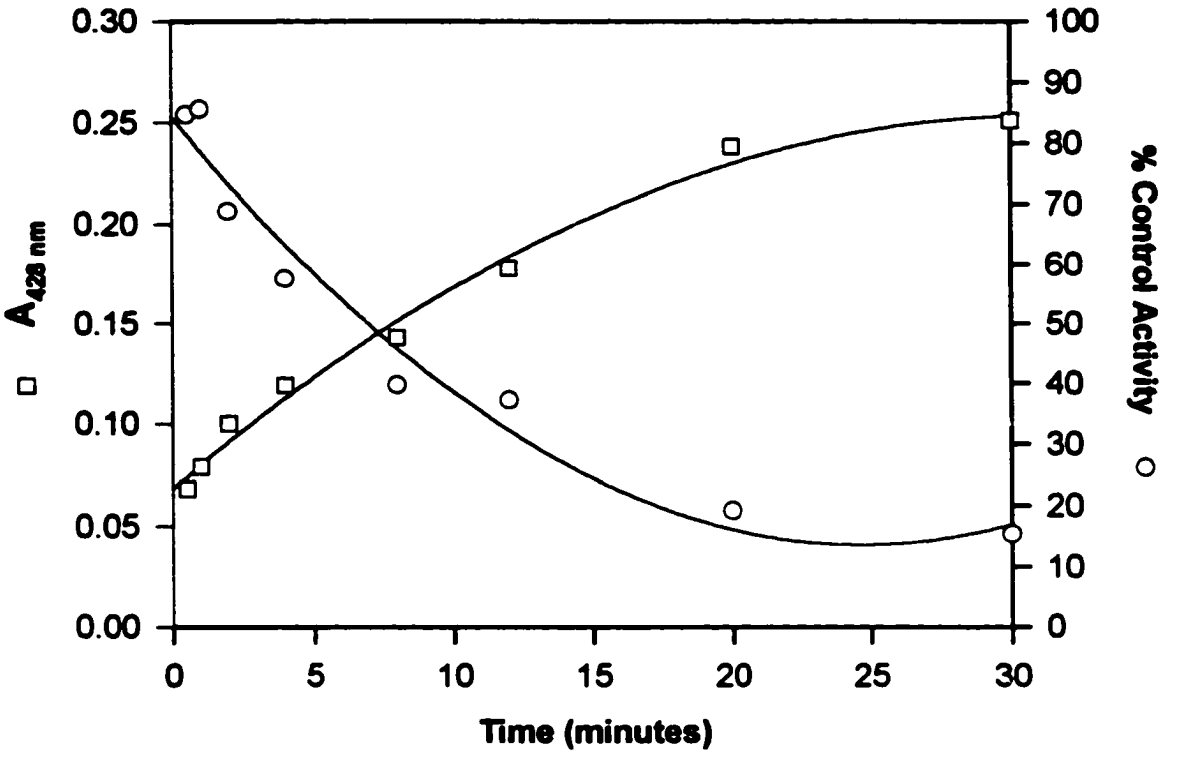


Figure 29: Correlation between the rate of TNM modification (□) and loss of HlyC acyltransferase actvity (o). S-tag-HlyC in 0.05 M Tris (pH 8.0) was treated with 1 mM TNM at 25 °C and at various time intervals 10 μg aliquots of HlyC were removed and assayed for activity (o). Activity is expressed as the percentage of a control reaction containing 10 μg of HlyC (45.0±0 pmoles acyl group transferred/min) that was not exposed to TNM. For absorbance studies, S-tag-HlyC modified under the same conditions was monitored for change in the absorbance at 428 nm.

tyrosine residue is important, but its functional –OH group is not.

Phenylglyoxal (PG) is relatively specific for arginine at neutrality giving derivatives containing two PG moieties per guanidino group (Figure 23 reaction 7) (*101*, *102*). Since HlyC contains two conserved arginines, residues 24 and 87, (Figure 22) the effects of PG on enzyme activity was tested. HlyC was sensitive to increasing concentrations of the reagent (Figure 24) with only 10±2 % of the control activity remaining following treatment with 5 mM PG, implicating an essential arginine residue.

The presence of substrate during chemical modification can be used to selectively prevent modification of residues that are somehow protected upon substrate binding. This protection is usually explained as the residue being located within the active site; however, protection may occur as a result of structural changes following substrate binding leading to a change in the accessibility of the residue to the chemical modifier (*103*). Protection against TNM-, DEPC-, and PG-induced inactivation of HlyC was examined using acyl-ACP and ACPSH. Acyl-ACP consistently offered significant protection from the various chemical modifiers, whereas ACPSH was unable to protect HlyC from TNM and DEPC modification, and offered little protection from PG (Figure 30).

Characterization of HlyC Mutants

Chemical modification of HlyC provided information for possible residues essential for the catalytic mechanism of the enzyme. In order, to examine the function of specific conserved residues and possibly corroborate the chemical findings, site-directed

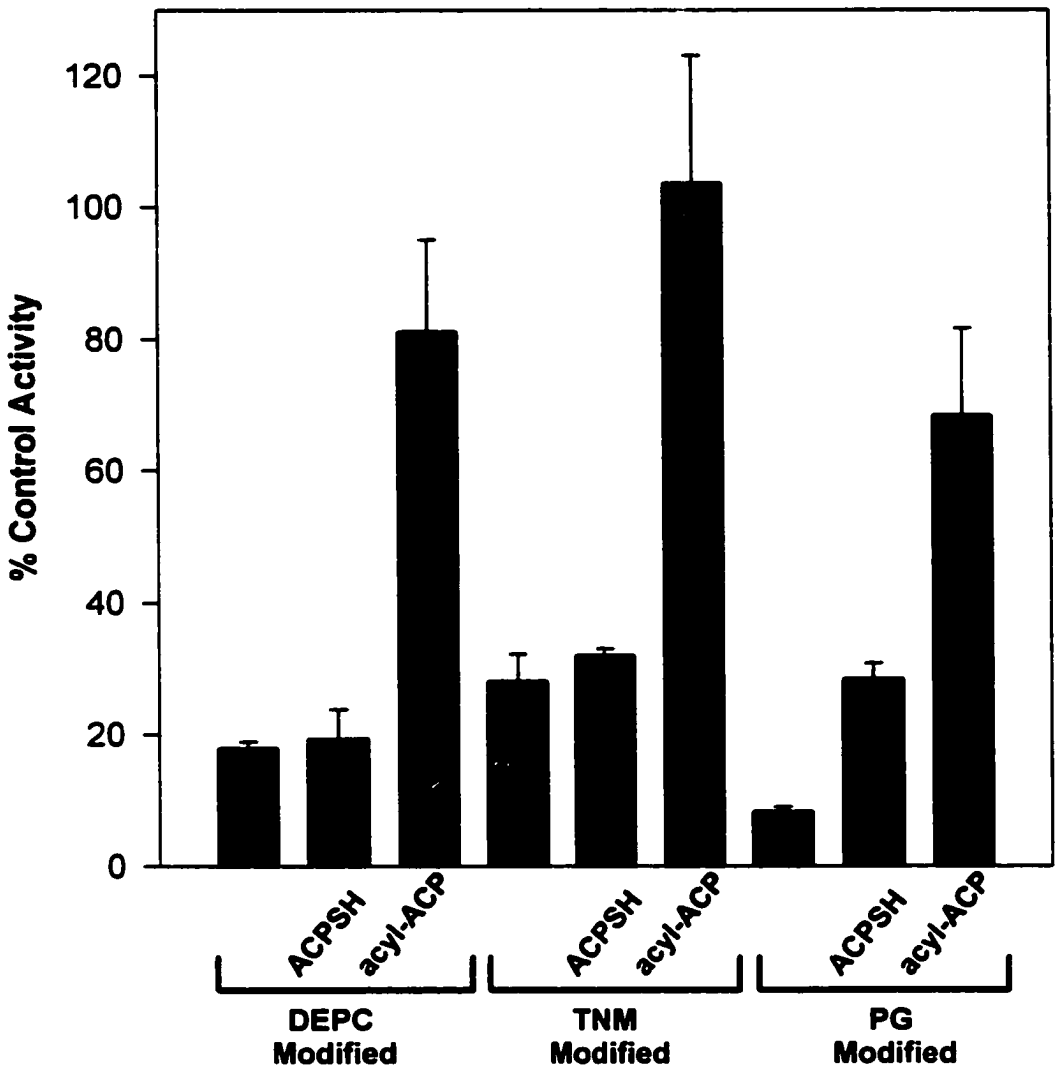


Figure 30: Protection of HlyC activity against DEPC, TNM, or PG inactivation by either acyl-ACP or ACPSH. Enzyme was modified with either 2 mM DEPC, 50-fold molar excess (0.22 mM) of TNM, or 5 mM PG. Prior to the addition of the chemical modifier, ~ 2 μM HlyC was incubated with either 2 μM unlabeled myristoyl-ACP or ACPSH. After modification, the unlabeled ACPs were removed by 70% $(NH_4)_2SO_4$ precipitation at pH 7.0 and the enzyme assayed using [^{14}C]myristoyl-ACP as described in Materials and Methods.

mutagenesis studies of the acyltransferase were employed. Residues that were conserved within the 13 different RTX toxin acyltransferases (Figure 22) were individually mutagenized, the mutation confirmed by DNA sequencing (data not shown), and the corresponding HlyC expressed and purified as a His_6-S-tag fusion protein. The expression of all HlyC mutants (data not shown) was identical to native HlyC (Figure 3). The acyltransferase activity was examined for mutant HlyCs isolated from inclusion bodies in HED 6 M urea followed by slow renaturation of the enzyme by dialysis (refolded HlyC) or for HlyC in which the urea concentration was lowered by dilution with HED buffer (quickly diluted HlyC). The initial activity studies for refolded and quickly diluted HlyCs are summarized in Table 8. Activites ranging from 0-10% of the native activity were indiscernible from 0 using the HlyC assay system presented, a common problem in assessing activities of mutant proteins (*104*).

Mutation of the conserved and only cysteine (Cys 57) of HlyC to an alanine showed the same level of acyltransferase activity as did wild-type enzyme. Changing conserved serines 58 and 76 of HlyC to alanines, showed little or no decrease in activity; however, the S20A mutant retained only ~30% wild-type activity. Interestingly, the S76A mutant experienced ~30% increase in activity resulting in native acyltransferase activity upon slow renaturation of the enzyme as compared to the quick diluted HlyC. Obviously, Ser 20 and 76 have some part in wild-type acyltransferase function.

HlyC was very sensitive to DEPC and TNM, leading to acetylation of histidine residues and nitration of tyrosine residues respectively. Mutation of the conserved

Table 8: ACYLTRANSFERASE ACTIVITES OF SINGLE-SITE MUTANT HLYCS

Mutation	% of Wild-Type Acyltransferase Activity[a]	
	Quick Diluted HlyC	Refolded HlyC
G11A	14.1±0.1	23.2±0.4
G85A	0.0±0.4	3.6±0.4
H23A	7.9±0.3	4.4±0.1
H23S	6.6±0.4	5.6±0.4
H23C	11.7±1.5	3.5±0.9
R24A	66.7±1.8	63.8±0.2
R87A	17.7±0.6	86.1±1.7
S20A	36.3±0.3	31.2±0.3
S58A	78.1±0.4	75.3±1.3
S76A	63.2±7.3	94.1±4.0
E67A	75.6±0.3	107.0±2.1
D86A	64.3±0.1	70.4±0.2
Y70G	10.0±1.1	11.8±0.1
Y70F	74.5±5.2	104.0±2.5
Y150G	21.1±1.2	44.3±0.7
Y150F	91.6±1.7	85.5±4.3
C57A	90.0±0.7	95.6±2.5

[a]Acyltransferase activities were measured as described in Materials and Methods. Slowly refolded wild-type HlyC activity was 71.0±2.3 picomoles acyl group transferred /5 μg enzyme. Wild-type quick dilution HlyC activity was 47.0±2.4 picomoles acyl group transferred/5 μg enzyme. Each % is the average of at least 3 determinations enzyme ± standard deviation.

histidine and the two conserved tyrosines significantly effected acyltransferase activity. Changing conserved His 23 to an alanine led to total inactivation of the acyltransferase. Mutation of conserved Tyr 70 and Tyr 150 to glycines resulted in inactivation of the acyltransferase for the Y70G mutant and 40% of the wild-type activity for the refolded Y150G mutant. Like the S76A mutant, the Y150G HlyC showed a difference in acyltransferase activity between the quickly diluted and refolded enzyme with a 20% increase upon slowly refolding the protein.

Mutation of conserved arginine residues 24 and 87 to alanines affected HlyC activity. The R24A HlyC mutant possessed ~65% of the wild-type activity for both the quickly diluted and refolded HlyCs; whereas, the R87A mutant demonstrated a significant difference between the two methods of renaturing HlyC. Quickly diluted R87A HlyC retained only ~20% of the wild-type activity, but upon slowly refolding it functioned at wild-type levels with ~90% activity. Becuase both R24A and R87A HlyCs possessed significant acyltransferase activities upon refolding, these mutants were tested for sensitivity to PG. Both HlyCs were still inhibited by PG to the same extent as the inhibition seen with the wild-type enzyme (data not shown), suggesting that both Arg 87 and Arg 24 could be the potential targets for PG modification as well as other arginines within HlyC.

No chemical modifications of HlyC were done that would modify acidic residues, but two mutants were made changing two conserved acidic residues to alanines. Changing Glu 67 to an alanine had no effect on enzyme activity. Changing Asp 86 to an alanine resulted in a 30-40% decrease in acyltransferase activity. These data suggest that neither residue is critical for HlyC activity, but Asp 86 does seem necessary for wild-type

acyltransferase function. Mutation of the two conserved glycines, Gly 11 and 85, to alanines were detrimental to acyltransferase activity resulting in 14-20% activity for mutant G11A and no activity for the G85A mutant.

To examine further the function of residues His 23, Tyr 70, and Tyr 150 these residues were mutated. Tyrosines 70 and 150 were mutated to phenylalanine. These HlyCs, Y70F and Y150F, functioned at native levels. Furthermore, both mutants were inactivated like the native enzyme by TNM (data not shown) therefore both Tyr 70 and 150 are potential targets for TNM along with other tyrosines in the enzyme. His 23 was also mutated to a serine and a cysteine residue, both of which could bear an acyl group. Like the H23A mutant, the H23S and H23C mutant acyltransferases showed no activity.

Mutant HlyCs were monitored for the formation of [^{14}C]acyl-enzyme intermediate and products resulting from chemcial cross-linking reactions with [^{14}C] myristoyl-ACP. The data are summarized in Figure 31, Figure 32, and Table 9. Essentially, any mutant HlyC that was able to function at or near wild-type levels produced [^{14}C]acyl-enzyme intermediate and cross-linked with [^{14}C]myristoyl-ACP like the native enzyme (Refer to Figure 12). Mutant HlyCs functioning in this manner included the following: R24A, C57A, S58A, E67A, Y70F, S76A, D86A, R87A, and Y150F. In some cases, single-site mutations of HlyC produced acyltransferases able to cross-link to acyl-ACP, but unable to produce acyl-HlyC intermediate. These mutations include H23A, H23C, H23S, S20A, G11A, Y70G, and Y150G. H23A, H23S, H23C, and S20A HlyCs all cross-linked at normal levels, while the G11A, Y70G, and Y150G mutants cross-linked at much lower levels as compared to the wild-type control. Only the G85A mutant resulted in a loss of heterodimer and acyl-intermediate formation. No

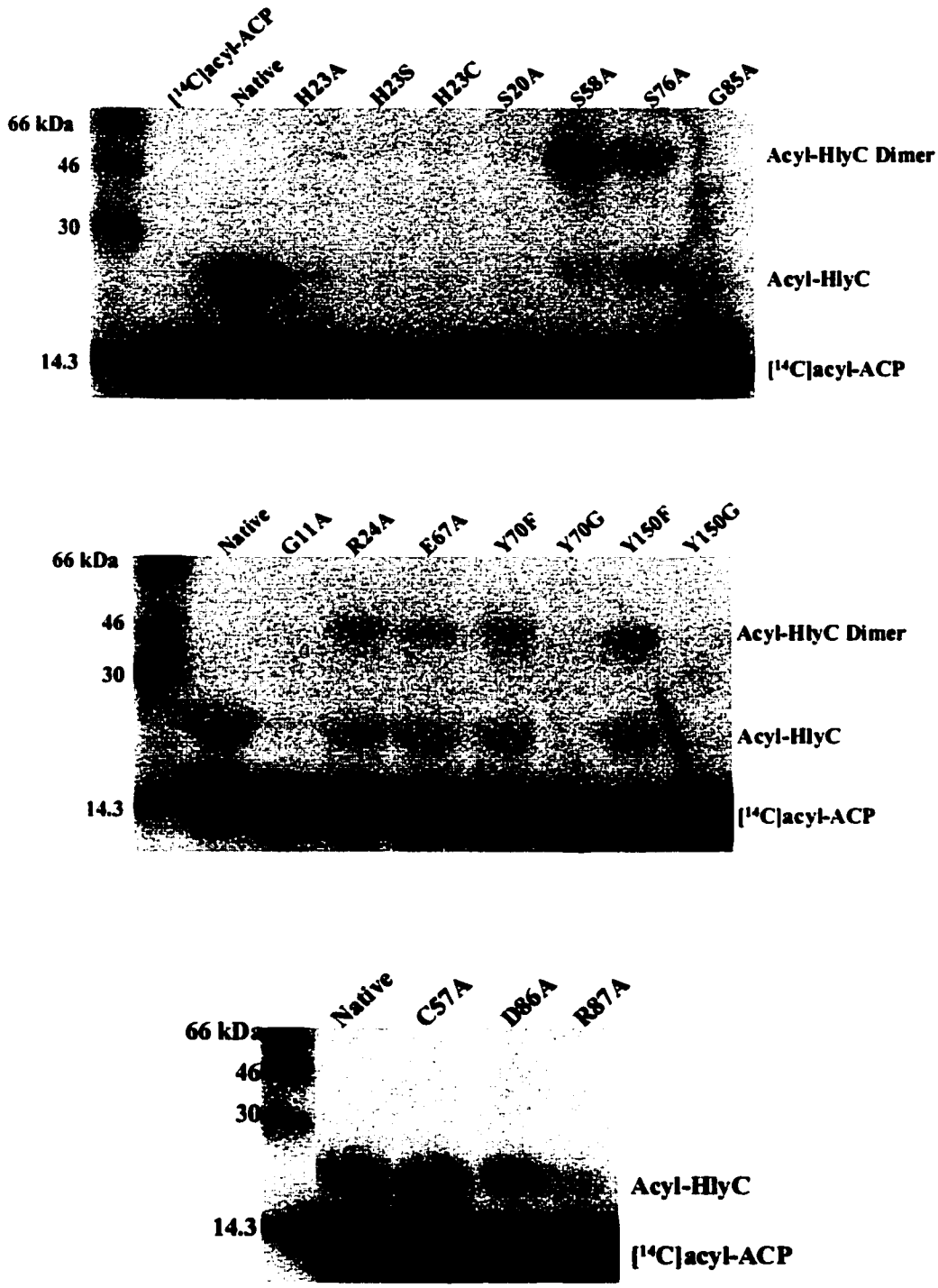


Figure 31: Fluorographic demonstration of [^{14}C]acyl-HlyC enzyme intermediate formation for mutant HlyCs. All reactions contained 2.5 μM [^{14}C]myristoyl-ACP

Figure 31 (continued): A reaction containing only [^{14}C]myristoyl-ACP is shown in the top fluorograph (negative control). When present in reaction, the particular HlyC is given above its respective lane. Reactions contained 50 μM of enzyme. Each fluorograph respresents a separate experiment and contains its own native control to which the mutant HlyCs were compared. Reactions were carried out as described in Materials and Methods.

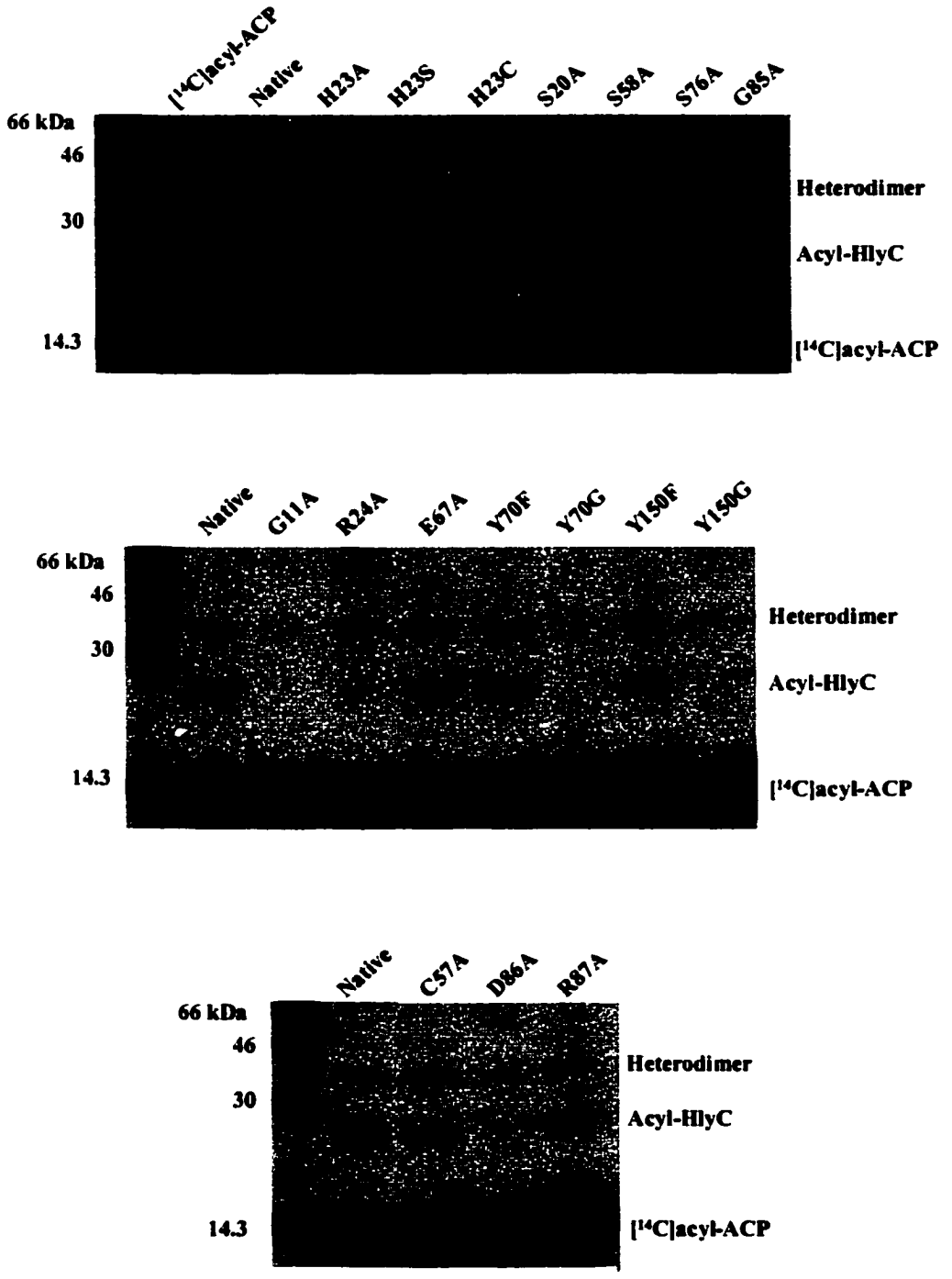


Figure 32: Fluorographic demonstration of Acyl-ACP-HlyC heterodimer formation upon chemical cross-linking with [^{14}C]myristoyl-ACP and mutant HlyCs in the presence of DMS

Figure 32 (continued): All reactions contained 2 μM [^{14}C]myristoyl-ACP and DMS. A reaction with 2 μM [^{14}C]myristoyl-ACP alone in the presence of DMS (negative control) is shown in the top fluorograph. When present in reaction, the particular HlyC is given above its respective lane. Reactions contained 10 μg (4.5 μM) of enzyme. Each fluorograph represents a separate experiment and contains its own native control to which the mutant HlyCs were compared. Reactions were carried out as described in Materials and Methods.

Table 9: SUMMARY OF ACYL-ENZYME INTERMEDIATE AND ACYL-ACP-HLYC HETERODIMER FORMATION OF HLYC MUTANTS[a]

Mutation	Acyl-Enzyme Formation	Acyl-ACP-HlyC Heterodimer Formation
G11A	Not Detectable	Low
G85A	Not Detectable	Not Detectable
H23A	Not Detectable	Normal
H23S	Not Detectable	Normal
H23C	Not Detectable	Normal
R24A	Intermediate	Intermediate
R87A	Low	Normal
S20A	Not Detectable	Normal
S58A	Normal	Normal
S76A	Normal	Normal
E67A	Normal	Normal
D86A	Intermediate	Intermediate
Y70G	Not Detectable	Low
Y70F	Normal	Normal
Y150G	Not Detectable	Low
Y150F	Normal	Normal
C57A	Normal	Normal

[a]Fluorographs from Figure 31 and 32 were scanned followed by densitometry measurements using the UnScan It Software. Densities of bands produced by mutant HlyCs were compared to bands produced by the native HlyC control on each fluorograph. Intensities of the bands were ranked as either normal, intermediate, low, or not detectable.

mutation produced an enzyme capable of acyl intermediate formation production with the lack of heterodimer production. For mutations S58A, S76A, R24A, E67A, Y70F, and Y150F an additional band was seen in acyl-enzyme formation experiments at the size expected for an acyl-HlyC dimer (Figure 31), whereas, wild-type enzyme never produced acyl-enyzme in the dimer form in any of the fluorographs.

The kinetic parameters for myristoyl-ACP and proHlyA were determined for His_6-S-tag-HlyC and mutant fusion proteins with detectable acyltransferase activity. Myristoyl-ACP had a K_m^{app} of 0.61±0.1 μM, and a V_{max}^{app} of 14.0±0.5 pmoles of acyl group transferred/min with proHlyA held constant at 2 μM (Table 10). ProHlyA had a K_m^{app} of 2.19±0.17 μM and a V_{max}^{app} of 15.2±2.7 pmoles acyl group transferred/min with myristoyl-ACP held constant at 1 μM (Table 11). There was no significant difference between the K_m^{app} values for myristoyl-ACP or proHlyA for the S-tag and His_6-S-tag HlyCs. Although the V_{max}^{app} value for myristoyl-ACP and proHlyA increased for the His_6-S-tag HlyC enzyme, the increase in acyltransferase activity was due to the improved quality of the proHlyA preparations rather than differences in the specific activity of the two fusion proteins.

For the majority of the single-site mutations there was no significant change in the value of K_m^{app} or V_{max}^{app} for either myristoyl-ACP (Table 10) or proHlyA (Table 11). This was not too surprising since through mutagenesis studies such as these it has become apparent that most proteins can tolerate single residue substitutions quite well (104). Of course the most interesting mutations were those that resulted in complete loss of function; G11A, H23A, H23S, H23C, Y70G, and G85A. There were, however, two

Table 10: KINETIC PARAMETERS OF MYRISTOYL-ACP FOR NATIVE AND MUTANT HIS_6-S-TAG-HLYCS[a]

Mutation	K_m^{app}	% of native K_m^{app}	V_{max}^{app}	% of native V_{max}^{app}
None	0.61±0.1	100	14.0±0.5	100
G11A	ND[b]	ND	ND	ND
G85A	ND	ND	ND	ND
H23A	ND	ND	ND	ND
H23S	ND	ND	ND	ND
H23C	ND	ND	ND	ND
R24A	0.32±0.02	52	6.2±0.1	44
R87A	0.36±0.10	59	7.0±0.3	50
S20A	0.40±0.10	66	1.8±0.7	13
S58A	0.26±0.01	43	5.6±0.2	40
S76A	0.30±0.01	49	7.6±0.1	54
E67A	0.77±0.01	126	17.0±0.7	121
D86A	0.29±0.01	48	9.2±0.1	66
Y70G	ND	ND	ND	ND
Y70F	0.80±0.10	131	11.8±0.4	84
Y150G	0.97±0.10	159	4.2±0.2	30
Y150F	0.65±0.10	107	14.2±0.5	101
C57A	0.62±0.01	102	13.7±0.6	98

[a]K_m^{app} in μM, and V_{max}^{app} is pmol incorporated/min. Kinetic parameters are apparent values for the particular conditions given including the concentration of the fixed substrate, proHlyA, which was 2μM. [b]Kinetic parameters of mutant HlyCs without sufficient activity were not determined (ND).

Table 11: KINETIC PARAMETERS OF PROHLYA FOR NATIVE AND MUTANT HIS_6-S-TAG-HLYCS[a]

Mutation	K_m^{app}	% of native K_m^{app}	V_{max}^{app}	% of native V_{max}^{app}
None	2.19±0.17	100	15.2 ±2.7	100
G11A	ND[b]	ND	ND	ND
G85A	ND	ND	ND	ND
H23A	ND	ND	ND	ND
H23S	ND	ND	ND	ND
H23C	ND	ND	ND	ND
R24A	2.12±0.13	97	10.6±0.3	70
R87A	2.61±0.26	119	11.6±0.6	76
S20A	1.31±0.10	60	2.0±0.1	13
S58A	1.43±0.15	65	7.13±0.3	47
S76A	2.39±0.20	109	12.5±0.5	82
E67A	1.89±0.13	86	15.0±0.5	99
D86A	1.78±0.10	81	10.5±0.3	69
Y70G	ND	ND	ND	ND
Y70F	1.89±0.13	86	15.0±0.5	99
Y150G	1.45±0.23	66	3.14±0.2	21
Y150F	1.71±0.06	78	12.6±0.2	83
C57A	2.20±0.17	100	14.6±0.6	96

[a]K_m^{app} in μM, and V_{max}^{app} is pmol incorporated/min. Kinetic parameters are apparent values for the particular conditions given including the concentration of the fixed substrate, myristoyl-ACP, which was 1μM. [b]Kinetic parameters of mutant HlyCs without sufficient activity were not determined (ND).

mutations that resulted in significant changes in the V_{max}^{app} values for both substrates. Both the Y150G and S20A HlyCs produced V_{max}^{app} values for myristoyl-ACP and proHlyA that were significantly reduced compared to the wild-type enzyme.

CHAPTER 4

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 (*15*, *105*) by showing that the purified fusion protein was the single factor necessary 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 4,450 moles/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 (*106*).

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, is not an appropiate measurement of acyltransferase activity since the stoichiometry of HlyA toxin lysis remains unkown. Futhermore, lysis was shown not to be a good measure of HlyA formation 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. Developing an HlyC assay system separate from toxin lysis is important for the study of the enzyme and its protein substrates. For example, Ca^{2+} has been shown to be required for binding the toxin to target cells (*30, 31*). 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 (*34*).

The effects 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 acyltransferase while the acyl-toxins exhibited yet a dissimilar ranking of power as lytic agents. Neither the lytic response nor 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 (*107*). Acyl-ACP efficacies declined with increasing chain length; there was no such trend in the lytic behaviors of HlyA's bearing different acyl groups. The introduction of double bonds into an acyl chain decreases its hydrophobicity and its affinity for membranes (*107*); 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 similar amounts of lysis. In contrast, the toxins acylated with 18 carbon chain length saturated and unsaturated fatty acids produced similar amounts of toxin and lysis. 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. 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 (*21*, *108*). 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 upon carbon chain length and unsaturation of the acyl-group, lysis without experimental qualification is not a valid measure of proHlyA activation. Furthermore, the identity of fatty acyl groups on HlyA produced *in vivo* are not known.

Acylation of proHlyA to form HlyA was more sensitive to fatty acyl-group structure than was lysis by the resulting HlyA, 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

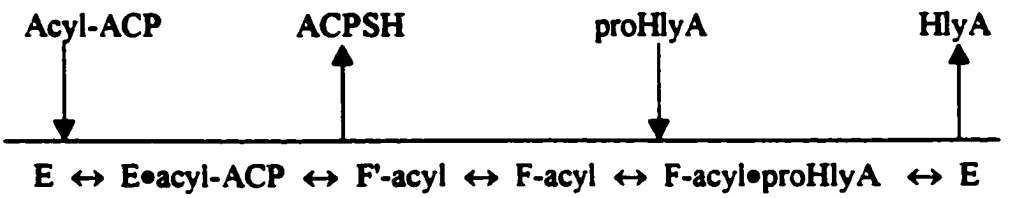
to different acyl chain lengths fitting an HlyC acyl group binding site for acyl-ACP. This hypothetical HlyC acyl group binding site likely operated secondary to protein-protein recognition in view of the fact that fatty acids alone were not inhibitory. Alternatively, slight but significant conformational 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 (*109*). Some degree of conformational variation among the acyl-ACPs studied is, however, established by their different migrations on conformationally sensitive non-denaturing PAGE in the presence of urea (*110*). The fatty acyl chain strengthens the native structure in proportion to the carbon chain length (*110*, *66*). A dynamic structure illustrated as a model involving two conformers provides the best fit of NMR analysis of ACP structure (*111*). 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. 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 non-reactive, dead-end product; it was a viable intermediate whose detection depended upon the lack of proHlyA, the acyl acceptor. Furthermore, the acyl-enzyme intermediate was able to catalyze the reverse reaction, transferring its acyl group to ACPSH. The formation of heterodimer, acyl-ACP-HlyC, shown by chemical cross-linking corroborated the HPLC demonstration that monomer was the active form of HlyC. The transfer of the acyl group from the acyl-

enzyme to proHlyA was apparent, with 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. The formation of the acyl-ACP-HlyC heterodimer, formation of an acyl-enzyme intermediate, and the fact that acyl-HlyC catalyzes the reverse reaction all support a substituted enzyme mechanism for the acyltransferase reaction.

The kinetic mechanism of HlyC was thoroughly investigated and the results were consistent with a uni uni iso uni uni ping pong kinetic mechanism where enzyme form F isomerizes (*91*). Scheme 1 summarizes the enzyme catalyzed reaction sequence in Cleland notation (*112*); F′ and F represent the two isomers of the enzyme.

Scheme 1: Kinetic Mechanism of HlyC, acyl-ACP-proHlyA acyltransferase.



Inhibition patterns generated by the products with respect to the different substrates enabled more exact definition of the substituted enzyme kinetic mechanism.

Inhibition by the first product, ACPSH, with respect to both myristoyl-ACP and proHlyA gave primary plots indicative of uncompetitive inhibition; replots of the $1/V_{max}^{app}$ values of the family of plots were, however, hyperbolic. Pure uncompetitive

inhibition would show a linear replot while the curves continued to move apart as [I] increased. Instead the inhibition was a mixed-type in which K_m and V_{max} changed by the same factor, but the displacement of the curves approached a limit as [I] increased giving a hyperbolic replot (*86*). In this situation, the product inhibitor combines with more than one enzyme form in alternate reaction sequences resulting in mixed-type inhibition, like that described generally by Cleland's rule 3 (*113*, *114*) and in more detail by Segel (*86*). Scheme 1 shows that the release of the product ACPSH generated an enzyme species different from that to which myristoyl-ACP bound. The enzyme species to which the second substrate, proHlyA, bound also differed from the enzyme form that released (and bound) ACPSH because of the isomerization of the F species of the enzyme prior to binding proHlyA. Inhibition by product under these circumstances would be expected to be mixed-type (*115*).

Inhibition by the second product, HlyA, unambiguously showed competitive inhibition in both primary plots with either myristoyl-ACP or proHlyA as varied substrates at unsaturating levels of the other. Replots of the data from the two separate primary plots of HlyA inhibition were, however, quite dissimilar indicating different mechanisms of inhibition by HlyA for each of the two substrates. The tendency of HlyA to aggregate (*76*) placed an upper limit on the concentrations that could be examined in the HlyC assay. With respect to myristoyl-ACP, HlyA was a parabolic or two site competitive inhibitor; the sites are separate and independent of one another (*87*). Replots to calculate K_i for a two site competitive inhibitor were not linear indicating that there were more than two independent inhibitor binding sites (*87*). Scheme 1 demonstrates that myristoyl-ACP and the product HlyA bind to the same species of enzyme thus predicting

a competitive type of inhibition in accordance with Cleland's rules that also predict the observed parabolic effect on replots upon binding of multiple inhibitor molecules (*113*).

HlyA inhibition with respect to proHlyA, although showing primary plots diagnostic of competitive inhibition, gave rise to replots quite different from parabolic or linear competitive inhibition. Both the hyperbolic replot of K_m^{app}/V_{max}^{app} against [I] and the leveling-off of v versus [I] curves indicated partial competitive inhibition (Figures 19 (inset) and 20B). Scheme 1, however, shows that, in contradiction to competitive inhibition, HlyA and proHlyA bind to different enzyme forms (*113*). Segel (*90*) describes multiple situations which give rise to mixed-type inhibition. Included is a circumstance that resembles partial competitive inhibition, but it is not. It is really a form of mixed-type inhibition, and it is a specific example of a Cleland rule 3 situation where a compound reacts with two different enzyme forms (sites) causing different effects which result in hyperbolic replots (*113*). According to the model presented by Segel (*116*) which is diagramed in Figure 33, the mixed-type inhibition in this instance arises from the hypothetical existence of two separate types of sites for inhibitor binding which are mutually exclusive. The model involves the interplay of two separate and differently behaving inhibitor binding sites and yet another binding site, the substrate binding site (Figure 33, E). Neither inhibitor binding site is the catalytic site (substrate binding site). There are, however, electronic or conformational responses within the enzyme upon occupation of either inhibitor binding site by inhibitor which cause the following behaviors in the remaining two sites, the alternate inhibitor binding site and the substrate binding site: One inhibitor binding site is a noninhibitory site that, when occupied,

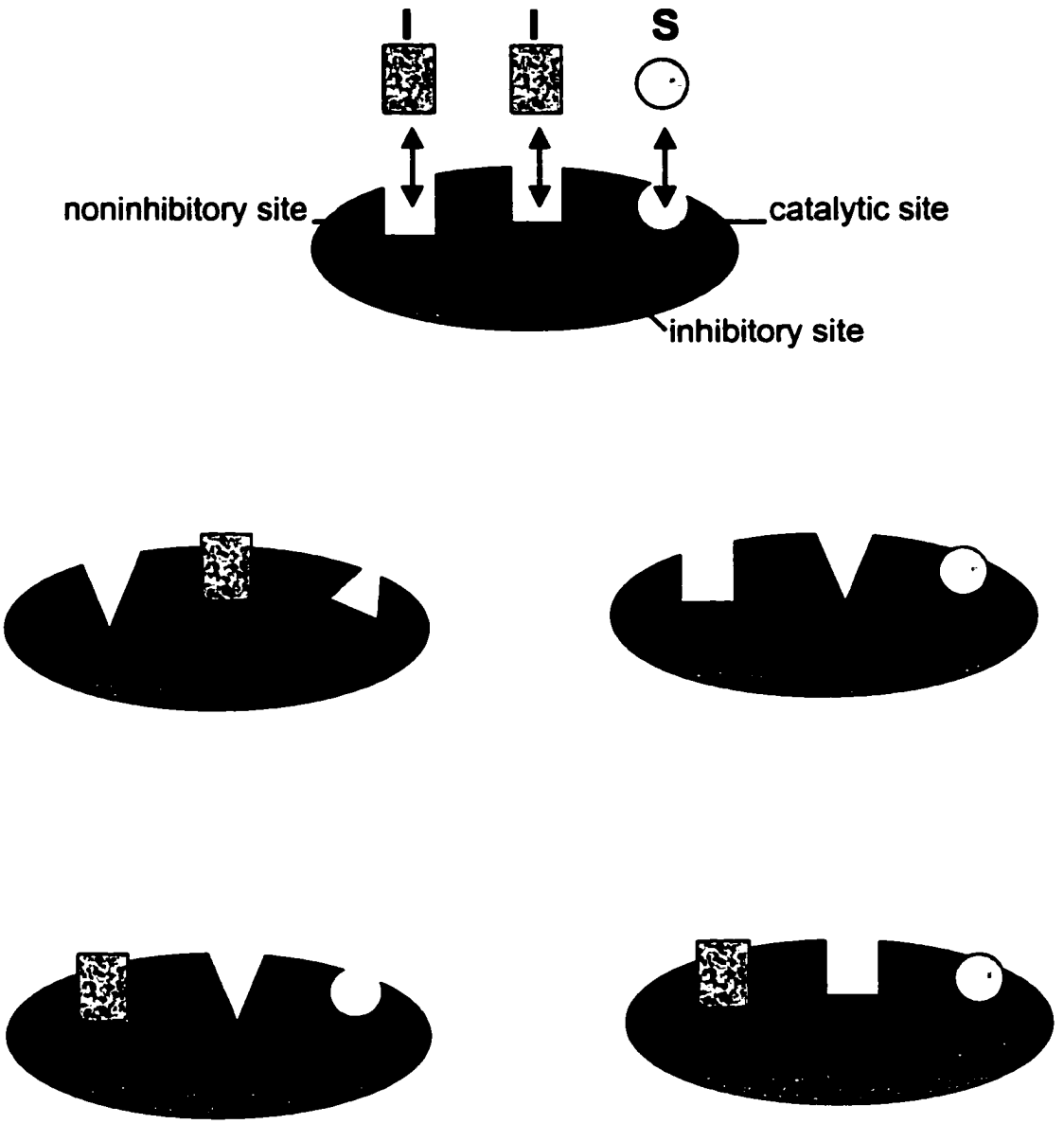


Figure 33: Mixed-type inhibition model of binding sites, possible occupancies, and resulting enzyme forms in HlyA product inhibition with respect to proHlyA. Abbreviations and symbols are as follows: I, inhibitor; S, substrate; EI*, inhibitory enzyme-inhibitor complex; IE, noninhibitory enzyme-inhibitory complex, ES, enzyme-substrate complex; IES, noninhibitory inhibitor-enzyme-substrate complex. Model adopted from Segel (*116*).

distorts the second inhibitor binding site and prevents its binding of inhibitor, while substrate binding (and subsequent activity) is unimpaired (Figure 33, IE and IES). Thus inhibition can only be partial because all inhibitor binding does not result in inhibition. The second inhibitor binding site, when occupied, causes distortions of both the noninhibitory inhibitor binding site and the substrate binding site (Figure 33, EI*). It prevents substrate from binding to the catalytic site (pure competitive action). The model gives rise to a mixed-type inhibition because in one instance inhibitor and substrate (IES) can bind simultaneously to the enzyme while in another instance only inhibitor (EI*) or substrate (ES) can bind to the enzyme. This mixed-type inhibition appears as partial competitive inhibition and cannot be distinguished by kinetics from a single-site model of partial competitive inhibition.

The steady state kinetics, the demonstration of a reactive acyl-enzyme intermediate, and the demonstration of the reversible first half reaction all supported a ping pong kinetic mechanism for HlyC. Distinctions among several different types of bi bi ping pong mechanisms were made by analyses of the product inhibition patterns with respect to the various substrates. The results of these studies of the internal protein acyltransferase reaction are compatible with a uni uni iso uni uni ping pong kinetic mechanism where enzyme form F isomerized.

Once the kinetic mechanism was defined, efforts were turned to elucidating the chemical mechanism of the enzyme. Chemical modification of HlyC suggested the possibility of histidines, tyrosines, and arginine residues being involved in HlyC catalysis while ruling out the possibility of any crucial serines, cysteines, and, indirectly, lysine residues. Overall, data from chemical modification and site-directed mutagenesis

experiments agreed very well. For example, the C57A mutant possessed wild-type acyltransferase function, and HlyC was insensitive to sulfhydryl modifying reagents. Also, treatment of the enzyme with TNM at pH 6.0 did not inactivate the enzyme, conditions that result in thiol oxidation (*117*). Mutation experiments revealed that Ser 20 and 76 were necessary for wild-type function. This finding did not contradict the chemical modification data showing no inhibition of acyltransferase activity by PMSF and AEBSF, since PMSF and AEBSF tend to react with unusually reactive serines and in some cases do not react with serines known to function as active site nucleophiles. For example, the malonyl-/acetyltransferase has an active site serine contained within the GXSXG motif characteristic of many serine active site esterases (*118*), but the enzyme is not inhibited by PMSF.

HlyC was very sensitive to DEPC modification and evidence was presented to suggest specific histidine modification. Inactivation of enzyme was enhanced at pH 6.0 which suggested histidine modification, since DEPC prefers unprotonated nucleophiles (*79*). Further evidence included the reactivation of DEPC modified HlyC by hydroxylamine treatment and the spectral evidence of N-carbethoxyhistidine formation with lack of ethoxyformylation of tyrosine. The importance of the modified histidine(s) was demonstrated by substrate protection experiments indicating the modified residue is located within or near the active site of HlyC. The fact that acyl-ACP protected HlyC from DEPC modification; whereas, ACPSH offered no protection could be attributed to a histidine lying within an acyl group binding site in the enzyme. Also, according to the predicted uni uni iso uni uni ping pong kinetic mechanism of HlyC, ACPSH binds to a different form of the acyltransferase than acyl-ACP; therefore, lack of protection could

simply be lack of protein-protein interaction of ACPSH with a specific form of the enzyme.

Although DEPC could modify any of the 6 histidines of HlyC, His 23 was chosen for site-directed mutagenesis experiments because of its conservation among the RTX toxin acyltransferases. Mutation of conserved His 23 to alanine corroborated the importance of histidine modification by DEPC. In addition to H23A, the H23S and H23C mutants were totally inactive. While serine and cysteine residues could function as nucleophiles during acyl-enzyme intermediate formation, they typically do not function in acid-base catalysis. Lack of activity for the H23S and H23C mutants does not rule out the possibility of His 23 functioning as the active site nucleophile since conversion of His 23 to a serine and cysteine residue could disturb the position and orientation of the active site. In some cases, substitution of the active site nucleophile with a second nucleophilic side chain is successiveful, but other times only minimal activity is retained. For example, the plant acyl-ACP thioesterase retains >60% activity upon mutation of the nucleophilic cysteine to a serine residue (*119*); whereas, replacement of the active serine of the malony-/acetyltransferase with cysteine results in retention of ~1% activity (*118*). Such a small retention of activity for H23C or H23S would not be detectable using the current HlyC assay system.

Inactivation of HlyC by TNM suggested an important tyrosine(s) and this was further evident by a pH dependency of TNM modification at pH 8.0, as well as spectral data showing formation of 3-nitrotyrosine. Chemical data suggesting a reduction in acyltransferase activity due to tyrosine modification was confirmed by mutating conserved Tyr 70 and 150 to glycines since mutational studies showed both residues were

critical for wild-type enzyme function. HlyC contains 7 tyrosines, 2 being fully conserved, and 3 more conserved for hydrophobicity. TNM could potentially modify all of HlyC's tyrosines, but only the function of Tyr 70 and 150 were addressed in the site-directed mutagenesis studies presented here since this work focused on conserved residues among the different RTX toxin acyltransferases. Substitution of a tyrosine to a glycine residue results in an amino acid change with the greatest decrease in hydrophobicity, but still allows for flexibility in protein secondary structure (*120*). Changing either Tyr 70 or Tyr 150 to a glycine residue did not predict changes in HlyC secondary structure. Comparison of quickly diluted and refolded HlyCs allowed further characterization of mutant enzymes helping to suggest roles for various residues. The 20% increase upon slowly refolding Y150G most likely results from conformational changes, suggesting Tyr 150 has a role in protein conformation and/or stabilization.

Decreases in activity observed with HlyC mutants Y70G and Y150G could easily be recovered by replacing the glycine with a phenylalanine restoring hydrophobicity and showing the lack of need for the –OH group. That an important tyrosine whose –OH group was not essential was also shown by chemical modification experiments, since HlyC acyltransferase activity was insensitive to acetylation of the tyrosyl –OH by NAI and acylation by DEPC (pH 8.0), but inactivated by TNM nitration of the tyrosyl phenolic ring (pH 8.0). As with DEPC inactivation of HlyC, the importance of a tyrosine residue(s) was underscored by protection experiments from TNM. Inactivation of enzyme by TNM in the presence of acyl-ACP was unsuccessful but in the presence of ACPSH inactivation proceeded as ususal. Perhaps one of the conserved tyrosine residues is necessary for providing a hydrophobic environment within an acyl group binding site

in HlyC for the acyl-ACP substrate. This may explain chemical cross-linking data since Y70G and Y150G formed low levels of heterodimer suggesting a role in substrate recognition. Also, mutation of the conserved tyrosines could lead to an overall conformational change decreasing acyl-ACP substrate recognition unrelated to acyl group recognition. It would be of interest to determine if ACPSH cross-links to a greater degree to Y70G and Y150G HlyC mutants, as compared to acyl-ACP.

HlyC contains 6 arginines with 2 conserved among the 13 acyltransferases. An important arginine(s) was suggested by PG modification and confirmed by mutation of conserved positively charged Arg 24 and Arg 87 to alanines. Like the Y150G mutant, R87A showed an increase in activity upon slow renaturation, in this case an increase of ~70% as compared to HlyC refolded by quick dilution. This data implied that Arg 87 is important in protein conformation. Reduction in activity seen with the R24A and R87A mutations could be a result in the removal of positive charge or possibly the change in residue size. Further mutations will be necessary to determine the role of these conserved residues. Acyl-ACP protected HlyC from PG modification, but to a lesser extent than found in TNM and DEPC modification experiments. Also for the first time ACPSH offered marginal protection. Taken together the protection experiments indicate that the modified arginine(s) is less likely to be influenced by the acyl chain of the acyl-ACP substrate and therefore less likely to be involved at the catalytic site. It seems that Arg 24 and 87 are most likely involved in a protein structural role rather than in the catalysis. Perhaps the positively charged residues participate in binding to the negatively charged acyl-ACP substrate, since ACPSH can protect HlyC from PG modification, albeit at

lower levels. This is partially supported by chemical cross-linking data since R24A produces less heterodimer compared to wild-type HlyC control.

Mutation of the only conserved acidic residues E67 and D86 to alanines gave mixed results. E67A functioned at wild-type levels, and D86A showed a decrease in activity. Finally conserved glycines 11 and 85 were mutated to alanines with both mutations leading to non-functional HlyCs, most likely arising from changes in protein conformation since glycine residues are typically not involved at the catalytic site. Whether or not a single-site amino acid change within HlyC could change the acyl group specificity of the acyl-ACP substrate was not addressed. Although this seems unlikely, since myristoyl-ACP was used in comparing mutant and native enzyme function, a change in the acyl chain specificity for a mutant enzyme as compared to the wild-type control would lead to misinterpretation of the results. Such an instance has been reported in which mutating a single amino acid of the lysophosphatidate acyltransferase (LPAT) resulted in changes in acyl-CoA substrate specificity (*121*).

Cross-linking experiments and acyl-enzyme intermediate formation by mutant HlyCs proved to be valuable experiments for suggesting roles of amino acid residues in the acyltransferase mechanism. For example, the H23A, H23S, and H23C mutants were incapable of forming acyl-enzyme intermediate, but all cross-linked at wild-type levels with acyl-ACP. These data imply His 23 is not necessary for acyl-ACP substrate binding, but critical for the formation of acyl-HlyC, the first half of the proHlyA acylation reaction. All single site mutations gave rise to HlyCs that cross-linked to acyl-ACP to some extent except for one, G85A. No mutants formed acyl-enzyme intermediate without cross-linking to acyl-ACP, suggesting that heterodimer formation is

a step along the pathway to acyl-enzyme formation. Upon examining acyl-intermediate formation for the various HlyCs, it was found that several enzymes produced an acyl-HlyC dimer, something not seen with the wild-type acyltransferase.

Determination of the kinetic parameters for the mutant enzymes showed little difference from the wild-type acyltransferase. There were, however, significant changes in V_{max}^{app} values for both acyl-ACP and proHlyA substrates when the S20A or Y150G mutants were the source of enzyme, implying that Ser 20 and Tyr 150 are involved in the the acyl transfer reaction. This, however, does not rule out a conformational role for Ser 20 and Tyr 150.

With the data from chemical modification and site-directed mutagenesis experiments one can conclude that HlyC contains several residues that are critical for acyltransferase function, an essential histidine; His 23, an essential tyrosine; Tyr 70, and two essential glycines; Gly 11 and 85. The remainder of the mutations, while in some cases significantly affecting enzyme function, were tolerable since HlyC was not completely inactivated. Of the residues listed above only His 23 and Tyr 70 could have an acyl group covalently attached to their respective side chains. Since replacement of the two conserved tyrosines with phenylalanines restored enzyme activity, Tyr 70 cannot be the site of HlyC acylation in acyl-intermediate formation. This leaves only His 23.

As discusssed earlier, based upon protein sequence comparison data, HlyC is a unique acyltransferase. This follows with the work presented here since HlyC appears to contain no essential cysteines or serines needed for acyl transfer, which is not the case for enzymes typically involved in these sort of reactions. For many acyltransferases, esterases, lipases, and proteinases a histidine residue functions in an acid-base role with a

second amino acid, a serine or cysteine, functioning as the nucleophile. Upon site-directed mutagenesis of these histidine residues, the enzymes become very inefficient, but, generally, complete loss of activity is seen only when the serine or cysteine residues that function as nucleophiles in the chemical mechanism are mutated (*122*). The HlyC acyltransferase is notable in that it loses all detectable activity upon mutation of the single conserved histidine residue, but the enzyme retains activity, although impaired in some instances, upon mutation of conserved serines or cysteine residues.

HlyC contains 1 cysteine and 11 serines. Since the C57A mutant retained native acyltransferase activity this removes the possibility of an –SH group functioning as a nucleophile. Although all of the serine residues were not mutated, with the chemical modification and mutagenesis data presented here it would seem unlikely that HlyC contains an active site serine since the remaining 8 serines are not conserved among the RTX toxin acyltransferases. To date, there have been no enzymes shown to possess a histidine functioning as the active site nucleophile giving rise to an acyl-imidazole intermediate. However, a histidine residue has been shown to act as a nucleophile forming an acyl-antibody intermediate in a reaction catalyzed by an antibody (*123*) and histidine 1106 of the C4B isotype of human complement forms an acyl-imidazole intermediate after attacking an internal thioester located within the protein (*124*, *125*). With the evidence presented above it is feasible that His 23 functions as a nucleophile in the transfer of acyl groups from acyl-ACP to proHlyA. In order to clarify further the chemical mechanism of HlyC, additional studies will be necessary.

BIBLIOGRAPHY

1. Grimminger, F., Sibelius, U., Bhakdi, Suttorp, N., and Seeger, W. (1991) *J. Clin. Invest. 88*, 1531-1539.

2. Bhakdi, S., Muhly, M., Korom, S., and Schmidt, G. (1990) *J. Clin. Invest. 85*, 1746-1753.

3. Welch, R. A. (1991) *Mol. Microbiol. 5*, 521-528.

4. Schmidt, H., Kernbach, C., and Karch, H. (1996) *Microbiol. 142*, 907-914.

5. Coote, J. G. (1992) *FEMS Microbiol. Rev. 88*, 137-162.

6. Goebel, W., and Hedgpeth, J. (1982). *J. Bacteriol. 151*, 1290-1298.

7. Nicaud, J.-M., Mackman, N., Gray, L., and Holland, I. B. (1985) *FEBS Lett.* 187, 339-344.

8. Hughes, C., Issartel, J-P., Hardie, K., Stanley, P., Koronakis, E., and Koronoakis, V. (1992) *FEMS Microbiol. Immunol. 105*, 37-44.

9. Vance, D. E., and Vance, J. (1996) *Biochemistry of Lipids, Lipoproteins and Membranes*, pp 41-42. Elsevier Science, New York.

10. Magnuson, K., Jackowski, S., Rock, C. O., and Cronan, J. E., Jr. (1993) *Microbiol. Rev. 57*, 522-542

11. Raetz, C. R. H. (1993) *J. Bacteriol. 175*, 5745-5753.

12. Rumley, M. K., Therisod, H. Weissborn, A. C., and Kennedy, E. P. (1992) *J. Biol. Chem. 267*, 11806-11810.

13. Jordan, S.W., and Cronan, J. E., Jr. (1997) *J. Biol. Chem. 272*, 17903-17906.

14. Issartel, J.-P., Koronakis, V., and Hughes, C. (1991) *Nature 351*, 759-761.

15. Hardie, K. R., Issartel, J.-P., Koronakis, E., Hughes, C., and Koronakis, V. (1991) *Mol. Microbiol. 5*, 1669-1979.

16. Stanley, P., Packman, L. C., Koronakis, V., and Hughes, C. (1994) *Science 266*, 1992-1996.

17. Ludwig, A., Garcia, F., Bauer, S., Jarchau, T., Benz, R., Hoppe, J., and Goebel, W. (1996) *J. Bacteriol. 178*, 5422-5430.

18. Hackett, M., Guo, L., Shabanowitz, J., Hunt, D. F., and Hewlett, E. L. (1994) *Sceince 266*, 433-435.

19. Menestrina, G., Moser, C., Pellet, S., and Welch R. (1994) *Toxicology 87*, 249-267.

20. Bakas, L., Ostolaza, H., Vaz, W. L. C., and Goni, F. M.(1996) *Biophys. J. 71*, 1869-1876.

21. Moayeri, M., and Welch, R. (1997) *Infect. Immun. 65*, 2233-2239.

22. Benz, R., Schmid, A., Wagner, W., and Goebel, W. (1989) *Infect. Immun. 57*, 887-895.

23. Menestrina, G., Moser, C., Pellett, S., and Welch, R. (1994) *Toxicology 87*, 249-267.

24. Suttorp, N., Fuhrmann, M., Tannert-Otto, S., Grimminger, F., and Bhakdi, S. (1993) *J. Exp. Med. 178*, 337-341.

25. Walev, I., Vollmer, P., Palmer, M., Bhakdi, S., and Rose-John, S. (1996) *Proc. Natl. Acad. Sci. USA 93*, 7882-7887.

26. Jonas, D., Schultheis, B., Klas, C., Krammer, P. H., and Bhakdi, S. (1993) *Infect. Immun. 61*, 1715-1721.

27. Mangan, D. F., Taichman, N. S., Lally, E. T., and Wahl, S. M. (1991) *Infect. Immun. 59*, 3267-3272.

28. Stanley, P., Koronakis, V., and Hughes, C. (1998) *Microbiol. Mol. Biol. Rev. 62*, 309-333.

29. Lally, E. T., Kieba, I. R., Sato, A., Green, C. L., Rosenbloom, J., Korostoff, J., Wang, J. F., Shenker, B. J., Ortlepp, S., Robinson, M. K., and Billings, P. C., (1997) *J. Biol. Chem.* 272, 30463-30469.

30. Boehm, D. F., Welch, R. A., and Synder, I. S. (1990a) *Infect. Immunol. 58*, 1951-1958.

31. Boehm, D. F., Welch, R. A., and Snyder, I. S. (1990b) *Infect. Immunol. 5*8, 1959-1964.

32. Ostolaza, H., Soloaga, A., and Goni, F. (1995) *FEBS Lett. 228*, 39-44.

33. Bakas, L., Veiga, M. P., Soloaga, A., Ostolaza, H., and Goni, F. M. (1998) *Bioch. Biophys. Acta 1368*, 225-234.\

34. Rose, T., Sebo, P., Bellalou, J., and Ladant, D. (1995) *J. Biol. Chem. 270*, 26370-26376.

35. Hewlet, E. L., Gray, L., Allietta, M., Ehrmann, I., Gordon, V. M., and Gray, M. C. (1991) *J. Biol. Chem. 266*, 17503-17508.

36. Olson, E. (1988) *Prog. Lipid Res. 27*, 177-197.

37. Johnson, D. R., Bhatnagar, R. S. Knoll, L. J., and Gordon, J. I., (1994) *Annu. Rev. Biochem. 63*, 869-914.

38. James, G., and Olson, E. N. (1990) *Biochemistry 29*, 2624-2634.

39. Schmidt, M. F. G. (1989) *Biochim. Biophys. Acta 988*, 411-426.

40. Das, A. K., Dasgupta, B., Bhattacharya, R., and Basu, J. (1997) *J. Biol. Chem. 272*, 11021-11025.

41. Liu, L., Dudler, T., and Gelf, M. H. (1996) *J. Biol. Chem. 271*, 23269-23276.

42. Camp, L. A., Verkruyse, L. A., Afendis, S. J., Slaughter, C. A., and Hofmann, S. L. (1994) *J. Biol. Chem. 269*, 23212-23219.

43. Stevenson, F. T., Bursten, S. L., Locksley, R. M., and Lovett, D. H. (1992) *J. Exp. Med. 176*, 1053-1062.

44. Stevenson, F. T., Bursten, S. L., Fanton, C., Locksley, R. M., and Lovett, D. H. (1993) *Proc. Natl. Acad. Sci. USA 90*, 7245-7249.

45. Vassilev, A. O., Plesofsky-Vig, N., and Brambl, R. (1995) *Proc. Natl. Acad. Sci. USA 92*, 8680-8684.

46. Hedo, J. A., Collier, E., and Watkinson, A. (1987) *J. Biol. Chem. 262*, 954-957.

47. Olson, E. N., Glaser, L., and Merlie, J. P. (1984) *J. Biol. Chem. 259*, 5364-5367.

48. Olson, E. (1988) *Prog. Lipid Res. 27*, 177-197.

49. Hackett, M., Walker, C. B., Guo, L., Gray, M. C., Van Cuyk, S., Ullmann, A., Shabanowitz, J., Hunt, D. F., Hewlett, E. L., and Sebo, P. (1995) *J. Biol. Chem. 270*, 20250-20253.

50. Forestier, C., and Welch, R. A. (1990) *Infect. Immun. 58*, 828-832.

51. Gygi, D. J., Nicolet, J., Frey, J., Cross, M., Koronakis, V., and Hughes, C. (1990) *Mol. Microbiol. 4*, 123-128.

52. Masure, H. R., Au, D. C., Gross, M. K., Donovan, M. G., and Storm, D. R. (1990) *Biochemistry 29*, 140-145.

53. Dobereiner, A. Shcmid, A., Ludwig, A., Goebel. W., and Benz, R. (1996*) Eur. J. Biochem. 240*, 454-460.

54. Ausubel, R.M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G. Smith, J. A., and Struhl, K. (1992) Short Protocols in Molecular Biology, 2nd edition, p. 1- 3, John Wiley & Sons, New York.

55. Novagen, *pET System Manual, 7th edition*, 1996.

56. Ausubel, R.M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G. Smith, J. A., and Struhl, K. (1992) *Short Protocols in Molecular Biology, 2nd edition*, p. 1-24, John Wiley & Sons, New York.

57. Koronakis, V., Cross, M., and Hughes, C. (1988) *Nucl. Acids Res. 16*, 4789-4800.

58. Trent, M. S., Worsham, L. M., and Ernst-Fonberg, M. L. (1998) *Biochemistry, 37*, 4644-4652.

59. Ausubel, R.M., Brent, R., Kingston, R. E., Moore, D. D., Siedman, J. G. Smith, J. A., and Struhl, K. (1992) *Short Protocols in Molecular Biology, 2nd edition*, p. 2-4 and 2-5, John Wiley & Sons, New York.

60. Schmidt, G., Selzer, J., Lerm., M., and Aktories, K. (1998) *J. Biol. Chem. 273*, 13669-13674.

61. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA. 74*, 5463-5467.

62. Walker, J. E., and Gay, N. S. (1983) *Methods Enzymol. 97*, 195.

63. Kim, J., and Raines, R. T. (1993) *Protein Science 2*, 348-356.

64. Kata, I., and Anfinsen, C.B. (1969) *J. Biol. Chem. 244*, 5849-5855.

65. Jackowski, S., Jackson, P. D., and Rock, C. O. (1994) *J. Biol. Chem. 269*, 2921-2928.

66. Rock, C. O., and Cronan, J. E., Jr. (1979) *J. Biol. Chem. 254*, 9778-9785.

67. Mohan, S., Kelley, T. M., Eveland, S. S., Raetz, C. R. H., and Anderson, M. S. (1994) *J. Biol. Chem. 269*, 3296-3303.

68. Vagelos, P. R., Majerus. P. W., Albert, A. W., Larrabee, A. R., and Ailhaud, G. P. (1966) *Fed. Proc. 25*, 1485-1494.

69. DiNello, R. K., and Ernst-Fonberg, M. L. (1973) *J. Biol. Chem.* 248, 1707-1711.

70. Bradford, M. M. (1976) *Anal. Biochem. 72*, 748-754.

71. Laemmli, V. (1970) *Nature 227*, 680-685.

72. Walker, T. A., Jonak, Z. L., Worsham, L. M. S., and Ernst-Fonberg, M. L. (1981) *Biochem. J. 199*, 383-392.

73. Wilkinson, G. N. (1961) *Biochem. J. 80*, 324-332.

74. Cornish-Bowden, A., and Eisenthal, R. (1974) *Biochem J. 139*, 721-730.

75. Soloaga, A., Ostolaza, H., Goni, F. M., and de la Cruz, F. (1966) *Eur. J. Biochem 238*, 418-422.

76. Ostolaza, H., Bartolome, B., de la Cruz, F., and Goni, F. M. (1991) *FEBS Lett. 280*, 195-198.

77. Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*. Holden-Day, Inc., San Francisco.

78. Riordan, J. F., and Vallee, B. L. (1967) *Methods Enzymol. 11*, 541-548.

79. Miles, E. W. (1977) *Methods Enzymol. 47*, 431-443.

80. Staab, H. A., and Rohr, W. (1968) *Newer Methods of Preparative Organic Chemistry*, p. 100 (of pp. 61-108), Academic Press.

81. Blight, M. A., Pimenta, A. L., Lazzaroni, J.-C., Dando, C., Kotlelvets, L., Seror, S. J., and Holland, I. B. (1994) *Mol. Gen. Genet. 245*, 431-440.

82. Worsham, L., Williams, S., and Ernst-Fonberg, M. L. (1993) *Biochim. Biophys. Acta 1170*, 62-71.

83. Purich, D. L. (1982) *Methods Enzymol. 87*,10.

84. Markus, M., Hess, B., Ottaway, J. H., and Cornish-Bowden, A. (1976) *FEBS Lett. 63*, 225-229.

85. Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*, pp 25-30, Butterworth & Company, London.

86. Segel, I. H. (1975) *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, p. 188, John Wiley & Sons, New York.

87. Segel, I. H. (1975) *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 465-469, John Wiley & Sons, New York.

88. Segel, I. H. (1975) *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 161-166, John Wiley & Sons, New York

89. Dixon, M., and Webb, E. C. (1979) *Enzymes 3rd Edition*, pp 353-354, Academic Press, New York.

90. Segel, I. H. (1975) *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 166-198, John Wiley & Sons, New York

91. Segel, I. H. (1975) *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 653-656, John Wiley & Sons, New York.

92. Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*, p. 19, Holden-Day, Inc., San Francisco.

93. Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*, p. 183, Holden-Day, Inc., San Francisco.

94. Riordan, J. F., and Vallee, B. L. (1967) *Methods Enzymol. 11*, 515-521.

95. Sokolovsky, M., Harell, D., and Riordan, J. F. (1969) *Biochemistry 8*, 4740-4745.

96. Christen, P., Vallee, B. L., and Simpson, R. T. (1971) *Biochemistry 10*, 1377-1384.

97. Sokolovsky, M., and Fuchs, M. (1970) *FEBS Lett. 7*, 167-170.

98. Doyle, R. J., Bello, J., and Roholt, O.A. (1968) *Biochim. Biophys. Acta 160*, 274-276.

99. Boesel, R. W., and Carpenter, F. H. (1970) *Biochem. Biophys. Res. Comm. 38*, 678-682.

100. Riordan, J. F., Wacker, W. E. C. and Vallee, B. L. (1965) *Biochemistry 4*, 1758-1763.

101. Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171-6179.

102. Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*, p. 195, Holden-Day, Inc., San Francisco.

103. Means, G. E., and Feeney, R. E. (1971) *Chemical Modification of Proteins*, pp 20-23, Holden-Day, Inc., San Francisco.

104. Axe, D. D., Foster, N. W., and Fersht, A. R. (1998) *Biochemistry 37*, 7157-7166.

105. Stanley, P., Koronakis, V., Hardie, K., and Hughes, C. (1996) *Mol. Microbiol. 20*, 813-822.

106. Speed, M. A., Wang, D. I. C., and King, J. (1996) *Nature Biotech. 14*, 1283-1287.

107. Peitzsch, R. M., and McLaughlin, S. (1993) *Biochemistry 32*, 10436-10443.

108. Pellett, S., and Welch, R. A. (1996) *Infect. Immun. 64*, 3081-307.

109. Jones, P.-J., Holak, T. A., and Prestegard, J. H. (1987) *Biochemistry 26*, 3493-3500.

110. Ohlrogge, J., Savage, L., Jaworski, J., Voelker, J., and Post-Beittenmiller, D. (1995) *Arch. Biochem. Biophys. 317*, 185-190.

111. Kim, Y., and Prestegard, J. H. (1989) *Biochemistry 28*, 8792-8797.

112. Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 104-137.

113. Cleland, W. W. (1963) *Biochim. Biophys. Acta 67*, 188-196.

114. Plowman, K. M. (1972) *Enzyme Kinetics*, pp 66-72, McGraw-Hill Book Co., New York.

115. Cornish-Bowden, A. (1979) *Fundamentals of Enzyme Kinetics*, pp. 76-77, Butterworth & Company, London.

116. Segel, I. H. (1975) *Enzyme Kinetics, Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*, pp 192-193, John Wiley & Sons, New York.

117. Riordan, J. F., and Valee, B. L. (1972) *Methods Enzymol. 25*, 515-521.

118. Vangipuram, S. R., and Smith, S. (1996) *J. Biol. Chem. 271*, 31749-31755.

119. Yuan, L., Nelson, B. A., and Caryl, G. (1996) *J. Biol. Chem. 271*, 3417-3419.

120. Kyte, J. (1995) *Structure in Protein Chemistry*, p. 60, Garland Publishing, Inc., New York.

121. Morand, L. Z., Patil, S., Quasney, M., and German, J. B. (1998) *Biochem. Biophys. Res. Comm. 244*, 79-84.

122. Rangan, V.S., and Smith, S. (1996) *J. Biol. Chem. 271*, 31749-31755.

123. Stewart J. D., Roberts, V. A., Thomas, N. R., Getzoff, E. D., and Benkovic, S. J. (1994) *Biochemisty 33*, 1994-2003.

124. Ren, X., Dodds, A. W., Enghild, J. J., Chu, C. T., and Law, S. K. A. (1995) *FEBS Lett. 368*, 87-91.

125. Dodds, A. W., Ren, X., Willis, A. C., and Law, S. K. A. (1996) *Nature 379*, 177-179.

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Trent, M. S., Worsham, L. M. S., and Ernst-Fonberg, M. L. 1998. HlyC, the Internal Protein Acyltransferase that Activates Hemolysin Toxin: Role of Conserved Histidine, Serine, and Cysteine Residues in Enzymatic Activity as Probed by Chemical Modification and Site-Directed Mutagenesis. *Biochemistry*. In Press.

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