# HlyC, the Internal Protein Acyltransferase That Activates Hemolysin Toxin: Roles of Various Conserved Residues in Enzymatic Activity As Probed by Site-Directed Mutagenesis†

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ABSTRACT: Hemolysin, a toxic protein produced by pathogenic *Escherichia coli*, is one of a family of homologous toxins and toxin-processing proteins produced by Gram-negative bacteria. HlyC, an internal protein acyltransferase, converts it from nontoxic prohemolysin to toxic hemolysin. Acyl-acyl carrier protein is the essential acyl donor. The acyltransferase reaction progresses through formation of a binary complex between acyl-ACP and HlyC to a reactive acyl-HlyC intermediate [Trent, M. S., Worsham, L. M., and Ernst-Fonberg, M. L. (1998) *Biochemistry 37*, 4644−4655]. The homologous acyltransferases of the family have a number of conserved amino acid residues that may be catalytically important. Experiments to illuminate the reaction mechanism were done. The formation of an acyl-enzyme intermediate suggested that the reaction likely proceeded through two partial reactions. The reversibility of the first partial reaction was shown by using separately subcloned, purified, and expressed substrates and enzyme. The effects of single site-directed mutations of conserved residues of HlyC on different portions of reaction progress (binary complex formation, acyl-enzyme formation, and enzyme activity, including kinetic parameters) were determined. Mutations of His23, the only residue essential for activity, formed normal binary complexes but were unable to form acyl-HlyC. The same was seen with S20A, a mutant with greatly impaired activity. Mutation of two conserved tyrosines separately to glycines results in greatly impaired binary complex and acyl-HlyC formation, but mutation of those residues to phenylalanines restored behavior to wild-type.

Internal fatty acylation of a protein is a means of modifying its biological behavior. For example, lipid modification of proteins influences protein−protein interaction, and it is a component of cell regulatory mechanisms such as signal propagation. Many toxins exert their effects by distortion of cellular signaling mechanisms. Numerous instances of protein internal fatty acylation, generally via thiol esterification of cysteine residues, have been reported. The acyltransferases catalyzing these reactions have been elusive. Recently, however, two protein−palmitoyl acyltransferases have been isolated (*1*, *2*). Although several instances of posttranslational modification by internal fatty acylation of specific proteins through amide linkage have been reported (*3* and references therein, *4*−*6*), the enzymes have been neither isolated nor characterized. The extent of internal fatty acylation via amide linkage of mammalian proteins is unknown. However, acylation of cellular protein with endogenously synthesized fatty acids in a mouse muscle cell line indicated that at least 30% of protein-bound palmitate was present in amide linkage with undefined residues (*7*).

The toxicity of hemolysin (HlyA),[1] a protein toxin secreted by pathogenic *Escherichia coli*, rests upon the acylation of two internal lysine $\epsilon$-amino groups. HlyA binds to and lyses mammalian target cell membranes and, at lower concentrations, perturbs cell signal transduction and release of inflammatory mediators (*8*, *9*). It is one of the RTX (repeats in toxin) family of homologous, membrane-active toxins of similar mechanism produced by different Gram-negative bacteria (*10*−*12*). The toxin arises from the expression of the *hlyCABD* gene cluster. The *hlyA* gene product, prohemolysin (proHlyA), is converted intracellularly to HlyA by the action of the *hlyC* gene product, HlyC, an internal protein acyltransferase (*13*−*16*). Acyl-ACP is the obligate acyl donor. Recently, we reported the separate subcloning and expression of each of the proteins involved in the internal acylation of proHlyA to form toxic HlyA and characterized the acyltransferase, HlyC, and the reaction it catalyzes (*16*). Notably, a reactive acyl-HlyC intermediate is formed (*16*),

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[1] Abbreviations: proHlyA, hemolysin A protoxin; HlyA, hemolysin A toxin; RTX, repeats in toxin; HlyC, acyl-acyl carrier protein−prohemolysin acyltransferase; ACP, acyl carrier protein; ACPSH, acyl carrier protein with a free prosthetic group thiol; myristoyl-ACP, acyl carrier protein with a 14-carbon acyl chain covalently attached to the prosthetic group thiol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol.

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Table 1: Primers Used to Construct HlyC Mutants

| name[a] | sequence (5′ to 3′)[b] |
|---|---|
| G11A upper | CCATTAGAGATTCTTGCTCATGTATCCTGGCTATGGGCCAG |
| G11A lower | CTGGCCCATAGCCAGGATACATGAGCAAGAATCTCTAATGG |
| P53A upper | CGGGATGATTACGCTGTCGCGTATTGTAGTTGG |
| P53A lower | CCAACTACAATACGCGACAGCGTAATCATCCCG |
| E67A upper | GGGCTAATTTAAGTTTAGAAAATGCTATTAAATATCTTAATGATG |
| E67A lower | CATCATTAAGATATTTAATAGCATTTTCTAAACTTAAATTAGCCC |
| G85A upper | GCAGAAGACTGGACTTCAGCTGATCGTAAATGGTTCATTGAC |
| G85A lower | GTCAATGAACCATTTACGATCAGCTGAAGTCCAGTCTTCTGC |
| D86A upper | CAGAAGACTGGACTTCAGGTGCTCGTAAATGGTTCATTGACTGG |
| D86A lower | CCAGTCAATGAACCATTTACGAGCACCTGAAGTCCAGTCTTCTG |
| P96A upper | GGTTCATTGACTGGATTGCTGCTTTCGGGGATAACGG |
| P96A lower | CCGTTATCCCCGAAAGCAGCAATCCAGTCAATGAACC |

[a] Letters are one letter amino acid abbreviations. [b] Mutant amino acid codons are underlined.

which suggests that the acyltransferase reaction may consist of two partial reactions as shown:

$$\text{acyl-ACP} + \text{HlyC} \leftrightarrow [\text{HlyC} \cdot \text{acyl-ACP}] \leftrightarrow \text{acyl-HlyC} + \text{ACPSH} \quad (1)$$

$$\text{acyl-HlyC} + \text{proHlyA} \leftrightarrow [\text{proHlyA} \cdot \text{acyl-HlyC}] \leftrightarrow \text{HlyA} + \text{HlyC} \quad (2)$$

$$\text{sum acyl-ACP} + \text{proHlyA} \leftrightarrow \text{HlyA} + \text{ACPSH}$$

However, neither the chemistry nor the kinetics of the mechanism of this reaction nor that of any other protein internal acyltransferase is known.

Comparison of deduced amino acid sequences of 13 RTX C proteins reveals extensive homology (*17*). Of the 170 residues in HlyC, 36 are identical among the known RTX C proteins. Among the conserved residues are several residues known to be catalytically important in other types of acyltransfer reactions. Likely a conserved residue is the site of acylation during the transient formation of acyl-enzyme intermediate. We have previously investigated, via chemical modifications and site-directed mutagenesis, the importance of conserved tyrosine, arginine, cysteine, serine, and histidine residues in HlyC catalysis (*18*, *19*). Here we report the effects of site-directed mutagenesis of additional conserved residues and the kinetic parameters of all mutants with measurable activity. The prospect that the reaction proceeded through two partial reactions was established by showing the reversibility of the first partial reaction, the formation of the acyl-HlyC intermediate. The abilities of the mutant HlyCs to form acyl-HlyC intermediates and chemically cross-linked heterodimers of myristoyl-ACP were examined.

## EXPERIMENTAL PROCEDURES

*Materials.* [1-$^{14}$C]Myristate and [1-$^{14}$C]sodium pantothenate were from New England Nuclear. Kodak film for fluorography was from Sigma. *Eco*RV, *Dpn*I, and Deep Vent DNA polymerase were from New England Biolabs. *PfuTurbo* DNA polymerase was from Stratagene. All chemicals were reagent-grade. Urea-containing buffers were always freshly prepared.

*Site-Directed Mutagenesis.* The construction of the HlyC fusion protein expression plasmid pTXC2 and single site-directed mutation of conserved serine, cysteine, histidine, arginine, and tyrosine residues have been described *(18, 19)*. *E. coli* strains were BL21(DE3)pLysS and NovaBlue from Novagen and XL2-Blue from Stratagene. Cells were grown in Luria broth except for expression of $His_6$-S-tag-HlyC and its mutants; these were grown in minimal medium, induced with 1 mM IPTG at $A_{600nm}$ 0.6, and harvested after 4−5 h. Oligonucleotides used for site-directed mutagenesis of *hlyC* were from Integrated DNA Technologies. The round circle PCR method described in the Quik-Change site-directed mutagenesis kit protocol (Stratagene) generated site-directed mutations in *hlyC* by using the plasmid pTXC2 as the reaction template. The rationale involved whole-plasmid PCR amplification with one set of primers for each mutation (shown in Table 1). Residual native plasmid was digested with the *dam* methylation-specific restriction endonuclease *Dpn*I, and the PCR product containing the mutation was transformed into XL2-Blue cells for efficient cloning of nonmethylated DNA. Plasmids containing mutant *hlyC*s were designated pTXC2 along with a description of the mutation and were transformed into BL21(DE3)pLysS cells for expression.

*Proteins.* Radiolabeled in the fatty acyl group and unlabeled myristoyl-ACP were prepared as described by Trent et al. (*16*). Myristoyl-ACP was purified and evaluated as described *(20)* and stored in aliquots at −80 °C. ACPSH was isotopically labeled by adding 2 $\mu$M [1-$^{14}$C]sodium pantothenate (0.6 $\mu$Ci) *(21, 22)* to 250 mL cultures of *E. coli* grown and processed to purify ACPSH as described (*16*). The purified ACPSH had a specific radioactivity of 0.3 Ci/mol. ProHlyA was hyperexpressed and purified as reported (*16*). A HlyC fusion protein, N-terminal $His_6$-S-tag-HlyC, was employed for site-directed mutagenesis (*18*). N-terminal $His_6$-S-tag-HlyC was extracted into 25 mM Hepes (pH 8.0), 5 mM EDTA, 1 mM DTT, and 6 M urea from inclusion bodies obtained from a 200 mL cell culture as described by Trent et al. (*16*). Both wild-type and mutant $His_6$-S-tag-HlyC inclusion body urea extracts were renatured by each of two methods, quick dilution and slow refolding, as previously described (*18*).

*Assays.* Protein was measured as described by Bradford (*23*). The purity of each protein used was assessed by SDS−PAGE (*24*). The HlyC-catalyzed transfer of radiolabeled acyl groups from acyl-ACP to proHlyA to form HlyA was measured as described previously (*16*). Fluorography of SDS−PAGE separations of radiolabeled proteins was done according to Trent et al. (*16*).

*Demonstration of Partial Reaction.* Detection of the reverse direction of the first partial reaction, the possible transfer of long-chain fatty acyl group from the intermediate, acyl-HlyC, to radiolabeled ACPSH was as follows: Under

the HlyC assay conditions described previously (*16*), unlabeled myristoyl-ACP, 25 $\mu$M, was incubated with either 25 (reaction 1) or 50 $\mu$M (reaction 2) HlyC for 10 min at 4 °C in order to form the reaction intermediate, unlabeled acyl-HlyC. The reverse reaction was begun by the addition of 25 $\mu$M [$^{14}$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 and then centrifuged at 13600*g* for 5 min. The protein pellet was washed with 500 $\mu$L of cold acetone. Precipitated protein was dissolved in 25 $\mu$L of 8 M urea + 20 $\mu$L of 2% SDS sample buffer and subjected to SDS−15% PAGE followed by fluorography as described previously (*16*). Control reactions, which were processed identically, included the following combinations: 25 $\mu$M myristoyl-ACP + 25 $\mu$M [$^{14}$C] labeled ACPSH; 50 $\mu$M HlyC + 25 $\mu$M [$^{14}$C] labeled ACPSH; and 25 $\mu$M [$^{14}$C] labeled ACPSH.

*Determination of Enzyme Kinetic Parameters.* Reaction velocities were measured by the assay described above, ensuring that rates were linear with time. Kinetic data were analyzed with a computer program previously described (*25*) that calculated kinetic parameters by several different methods and by the nonlinear regression analysis of Wilkinson (*26*). The different methods of computing kinetic constants agreed; all gave $R$ values greater than 0.97. Reported kinetic constants were those computed from the direct linear analyses (*27*).

## RESULTS

*Site-Directed Mutagenesis of Single Residues of HlyC.* Extensive homology exists among the deduced amino acid primary sequences of the 13 RTX toxin C proteins that have been identified (Figure 1). Of 170 residues in HlyC, 36 are identical in all the RTX toxin C proteins, and still more are conserved as residue type. The kinetic or chemical mechanism is not known for any internal protein acyltransferase; an active acyl-enzyme intermediate, however, has been shown for the acyltransferase HlyC that activates *E. coli* hemolysin toxin (*16*). Conserved amino acid residues in HlyC that could bear an acyl group during an acyl transfer involving an acyl-enzyme intermediate include the following possibilities: those with an alcohol group, serines (three conserved) or tyrosines (two conserved); a sulfhydryl group, cysteine (one conserved); an imidazole group, histidine (one conserved); residues with an acid group (two conserved); and possibly, but unlikely, a ureido group, arginine (one conserved). No lysine residues were conserved among all 13 proteins. Chemical modification and site-directed mutagenesis of many of these residues, one residue at a time, was done in order to probe their importance in catalysis. These studies indicate that particular conserved serine and tyrosine residues are important for catalysis and that His23 is absolutely essential for catalysis (*18, 19*).

Possible roles of other conserved residues have been investigated by additional site-directed mutagenesis of single residues. The following site-directed mutations of N-terminal His$_6$-S-tag-HlyC were confirmed by DNA sequence analysis: G11A, P53A, G85A, E67A, D86A, and P96A. The mutant HlyCs expressed and purified like the wild-type His$_6$-S-tag-HlyC *(18)*. Of the three conserved glycine residues, two that were near clusters of other conserved amino acids were mutated separately to alanine residues, G11A and G85A, and each mutation resulted in greatly impaired or no acyltransferase activity (Table 2). Glu67 is identical in all RTX C proteins while Asp86 is conserved as an acidic residue. Mutation of each of these acidic residues to an alanine resulted in about 70% of wild-type enzyme activity upon refolding by quick dilution (Table 2). When E67A was slowly refolded by dialysis, its activity was indistinguishable from that of wild-type HlyC, while D86A activity remained impaired. Two of the three conserved prolines in HlyC occurred within clusters of other conserved amino acids in the primary sequence and were changed to alanine residues. P53A and P96A each resulted in diminished acyltransferase

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                20                  30                  40                  50
GHVSWLWASS  PLHRNWPVSL  FAINVLPAIQ  ANQYVLLTRD

                60                  70                  80                  90
DYPVAYCSWA  NLSLENEIKY  LNDVTSLVAE  DWTSGDRKWF

               100                 108                 118                 127
IDWIAPEGDN  - - GALYKYMR  KKFPDELFRA  IRVDPKTH - V

               137                 147                 157
GKVSEFHGGK  IDKQLANKIF  KQYHHELITE
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FIGURE 1: Amino acids conserved among 13 RTX toxin C proteins. Residues numbers refer to *E. coli* HlyC encoded by pHly 152. Nonconserved amino acids are shown in uppercase letters; conserved residues are shown in boldface uppercase letters. Amino acids that are identical in all 13 RTX C proteins are shaded. Breaks introduced to maximize homology are indicated by dashes. N and C termini, which are not shown, contain no conserved residues. Amino acid sequences were deduced from the following DNA sources: HLYC_ECOLI, *E. coli* HlyC, pHly152-encoded, accession no. P06736; HLC1_ECOLI, *E. coli* strain J96 HlyC, chromosome-encoded, accession no. P09984; HLC2_ECOLI, *E. coli* strain 200*l* HlyC, chromosome-encoded, accession no. p09985; HLYCEH, *E. coli* 0157:H7 strain EDL 933 HlyC, plasmid-encoded, translation of nucleotide sequence accession no. X80891; RT1C_ACTPL, *Actinobacillus pleuropneumoniae* Apx-IC, accession no. P55132; RT2C_ACTPL, *A. pleuropneumoniae* Apx-IIC, accession no. P15376; RT3C_ACTPL, *A. pleuropneumoniae*, Apx-IIIC, accession no. Q04474; HLYC_ACTAC, *Actinobacillus actinomycetemcomitans* LktC, accession no. P16461; HLC1_PASHA, *Pasteurella haemolytica* serotype A1 LktC, accession no. P16533; HLC3_PASHA, *P. haemolytica* serotype 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. The sequence alignment was done with the Hibio Prosis protein analysis software by Hitachi.

Table 2: Acyltransferase Activities of HlyC Single-Site Mutants[a]

| | % of wild-type acyltransferase activity | |
|---|---|---|
| mutant | quick dilution | slowly refolded |
| G11A | 14 ± 0.1 | 23 ± 0.4 |
| P53A | 35 ± 1.5 | 77 ± 5.2 |
| G85A | 0.0 ± 0.4 | 3.6 ± 0.4 |
| E67A | 75 ± 0.3 | 110 ± 2.1 |
| D86A | 64 ± 0.1 | 70 ± 0.2 |
| P96A | 23 ± 3.0 | 49 ± 6.4 |

[a] Acyltransferase activity was measured as previously described (*16*). Quick dilution and slow refolding renaturation of HlyC were done as previously described (*19*). Slowly refolded wild-type His$_6$-S-tag-HlyC activity was 2.8 ± 0.09 nmol of acyl group transferred min$^{-1}$ (mg of enzyme)$^{-1}$. Quickly diluted wild-type His$_6$-S-tag-HlyC activity was 1.9 ± 0.10 nmol of acyl group transferred min$^{-1}$ (mg of enzyme)$^{-1}$. Data are the averages of at least three determinations of enzyme activity ± the standard deviation.

Table 3: Kinetic Parameters of $His_6$-S-tag-HlyC Mutants with Measurable Activity[a]

| mutation | acyl-ACP $K_m^{app}$ | acyl-ACP $V_{max}^{app}$ | proHlyA $K_m^{app}$ | proHlyA $V_{max}^{app}$ |
|---|---|---|---|---|
| none | 0.61 ± 0.1 | 2800 ± 100 | 2.2 ± 0.2 | 3000 ± 540 |
| S20A | 0.40 ± 0.1 | 360 ± 140 | 1.3 ± 0.1 | 400 ± 20 |
| R24A | 0.32 ± 0.0 | 1200 ± 20 | 2.1 ± 0.1 | 2100 ± 60 |
| C57A | 0.62 ± 0.0 | 2700 ± 120 | 2.2 ± 0.2 | 2900 ± 120 |
| S58A | 0.34 ± 0.0 | 1200 ± 21 | 1.4 ± 0.2 | 1400 ± 60 |
| E67A | 0.77 ± 0.0 | 3400 ± 140 | 1.9 ± 0.1 | 3000 ± 80 |
| Y70F | 0.80 ± 0.1 | 2400 ± 80 | 1.9 ± 0.1 | 3000 ± 100 |
| S76A | 0.30 ± 0.0 | 1500 ± 20 | 2.4 ± 0.2 | 2500 ± 100 |
| D86A | 0.29 ± 0.0 | 1800 ± 20 | 1.8 ± 0.1 | 2100 ± 60 |
| R87A | 0.36 ± 0.1 | 1400 ± 60 | 2.6 ± 0.3 | 2300 ± 120 |
| R87K | 0.58 ± 0.1 | 2800 ± 120 | 1.4 ± 0.2 | 2400 ± 110 |
| Y150G | 0.97 ± 0.1 | 840 ± 40 | 1.5 ± 0.2 | 630 ± 40 |
| Y150F | 0.65 ± 0.1 | 2800 ± 100 | 1.7 ± 0.1 | 2500 ± 40 |

[a] For the determination of kinetic constants at varying concentrations of myristoyl-ACP, proHlyA was 2 $\mu$M; when proHlyA was the varied substrate, myristoyl-ACP was 1 $\mu$M. $K_m^{app}$ values are micromolar, and $V_{max}^{app}$ values are picomoles of myristate transferred per minute per milligram of enzyme. $K_m^{app}$ and $V_{max}^{app}$ values are to 2 significant numbers ± standard deviation. Experimental details and the means of calculating the kinetic constants and error estimates are given in Experimental Procedures. Kinetic parameters were not determined for the following mutant HlyCs, which were virtually inactive: G11A, H23A, H23S, H23C, Y70G, and G85A. Kinetic parameters were not determined for R24K, a mutant that had wild-type activity *(18)*, nor were they determined for the separate site-directed mutations of conserved proline residues, P53A and P96A. The generation of $His_6$-S-tag-HlyC mutants is in Experimental Procedures or was previously described (*18, 19*).

activity compared to wild-type enzyme (Table 2).

Kinetic parameters for many of the above mutants and single site-directed mutations that were previously described in conjunction with chemical modification studies are shown in Table 3 (*18, 19*). For each mutation, the ratios $V_{max}^{app}/K_m^{app}$, determined for each substrate, were used as estimates of the reaction rate constant in order to compare the effects of the various mutations on catalytic efficiency; the comparisons are given as percentages of the wild-type values (Table 4). In addition to mutations of each of the two conserved glycine residues, separate mutations of the single conserved His23 to an alanine, cysteine, or serine residue or one of the two conserved tyrosines, Tyr70, to a glycine residue resulted in complete loss of acyltransferase activity (Table 4). Mutation of the other conserved tyrosine, Tyr150, to a glycine resulted in greatly reduced enzyme activity stemming mainly from decreased $V_{max}^{app}$ values (Table 3). Replacement of either conserved tyrosine with a large, hydrophobic phenylalanine residue giving Y70F or Y150F fully restored activity of the slowly refolded enzyme to wild-type levels except for Y70F myristoyl-ACP substrate dependency (Table 3). Its $K_m^{app}$ was within wild-type limits, while $V_{max}^{app}$ was reduced, resulting in a rate of about 60% of the wild-type value. Other mutants that showed impaired activity include the following (Tables 3 and 4): S20A catalytic rate was about 20% of that of wild-type enzyme; the reduced catalysis stemmed mainly from a greatly lowered $V_{max}^{app}$. Of the other two mutations of conserved serine residues, S76A catalysis approached that of wild-type, and S58A showed decreased $V_{max}^{app}$ values. Arginine mutants R24A and R87A had diminished rates of catalyses due to decreased $V_{max}^{app}$ values. Replacement of Arg87 with another positively charged residue, lysine, resulted in an enzyme with wild-type catalytic efficiency. Kinetic parameters for a similar replacement of Arg24, R24K, were not measured since its specific activity was identical to that of wild type upon quick dilution refolding (*19*).

*Reversibility of Acyl-Enzyme Intermediate Formation.* The previous demonstration of the kinetically active acyl-enzyme intermediate in the HlyC-catalyzed reaction (*16*) suggests that the acyltransferase reaction may represent the sum of two partial reactions as follows:

$$\text{acyl-ACP} + \text{HlyC} \leftrightarrow [\text{HlyC} \cdot \text{acyl-ACP}] \leftrightarrow \text{acyl-HlyC} + \text{ACPSH} \quad (1)$$

$$\text{acyl-HlyC} + \text{proHlyA} \leftrightarrow [\text{proHlyA} \cdot \text{acyl-HlyC}] \leftrightarrow \text{HlyA} + \text{HlyC} \quad (2)$$

$$\text{sum acyl-ACP} + \text{proHlyA} \leftrightarrow \text{HlyA} + \text{ACPSH}$$

Partial reaction 1 was shown to be an integral entity by demonstrating the ability of the enzyme to catalyze partial reaction 1 in the reverse direction. This was done by providing radiolabeled ACPSH to unlabeled acyl-HlyC and documenting the formation of radiolabeled acyl-ACP. Unlabeled myristoyl-ACP and HlyC were incubated together for 10 min at 4 °C in order to form unlabeled myristoyl-HlyC, one of the two substrates in the reverse of partial reaction 1. Then [$^{14}$C]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, dissolved, and separated by SDS−PAGE. The formation of myristoyl-[$^{14}$C]ACP from unlabeled myristoyl-HlyC and [$^{14}$C]ACPSH is shown in Figure 2, lanes 6 and 7. Comparisons of the radiolabeled ACPSH band densities in lanes 4 and 5 with those in lanes 6 and 7 and the appearance of the myristoyl-ACP bands in lanes 6 and 7 indicate that a substantial amount of ACPSH was converted to myristoyl-ACP in the 10 min incubation at 4 °C. Twice as much HlyC was used to generate myristoyl-HlyC in reaction 2 (lane 7) compared to reverse reaction 1 (lane 6), and a larger amount of myristoyl-[$^{14}$C]ACP was formed in reverse reaction 2. Although myristoyl-HlyC was not isolated prior to demonstration of the reverse partial reaction, the extent of its formation has been shown to be proportional to the amount of HlyC present (*16*); thus the extent of the reverse reaction was related to the amount of myristoyl-enzyme formed. An assay that contained only myristoyl-ACP and radiolabeled ACPSH (lane 5) showed no labeled myristoyl-ACP, indicating that 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). HlyC in the form of acyl-HlyC catalyzed the proposed partial reaction 1 in the reverse direction; consequently ACPSH was converted to acyl-ACP.

*Acyl-Enzyme Formation and Chemical Cross-Linking Capabilities of HlyC Mutants. A*n acyl-enzyme intermediate is formed in the wild-type HlyC-catalyzed reaction as the product of partial reaction 1 above, and as previously shown, it is demonstrable in the absence of the second substrate proHlyA (*16*). The abilities of the different mutants to generate an acyl-enzyme intermediate were examined (Figure 3 and Table 4). Mutants that had no detectable acyltransferase activity showed no acyl-enzyme intermediate formation. These were G11A, H23A, H23S, H23C, and G85A. In

Table 4: Summary of Catalytic Capabilities of HlyC Mutants Compared to Wild Type[a]

| mutation | acyl-ACP × HlyC heterodimer formation | acyl-HlyC formation | % wild-type acyl-ACP $V_{max}^{app}/K_m^{app}$ | % wild-type proHlyA $V_{max}^{app}/K_m^{app}$ |
|---|---|---|---|---|
| G11A | low | not detected | inactive | inactive |
| S20A | normal | not detected | 20 | 22 |
| H23A | normal | not detected | inactive | inactive |
| H23C | normal | not detected | inactive | inactive |
| H23S | normal | not detected | inactive | inactive |
| R24A | normal | normal | 83 | 71 |
| C57A | normal | normal | 96 | 93 |
| S58A | normal | normal[b] | 78 | 71 |
| E67A | normal | normal[b] | 96 | 110 |
| Y70F | normal | normal | 65 | 110 |
| Y70G | low | not detected | inactive | inactive |
| S76A | normal | normal[b] | 110 | 79 |
| G85A | not detected | not detected | inactive | inactive |
| D86A | reduced | reduced[b] | 130 | 86 |
| R87A | reduced | low | 85 | 64 |
| R87K | reduced | normal[b] | 100 | 120 |
| Y150F | normal | normal[b] | 96 | 107 |
| Y150G | low | not detected | 20 | 31 |

[a] Data taken from Table 3 and Figures 3 and 4. [b] Estimate included acyl-HlyC monomer and dimer.

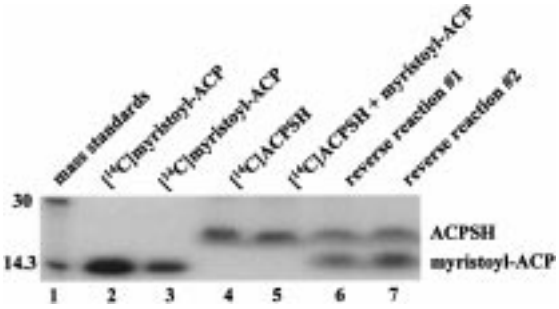


FIGURE 2: Fluorographic demonstration of the reversibility of partial reaction 1. Protein mixtures were prepared, treated, analyzed by SDS−PAGE, and subjected to fluorography as described in Experimental Procedures. The following proteins were applied to lanes 1−3: lane 1, radioactive mass standards given in kilodaltons; lane 2, [$^{14}$C]myristoyl-ACP, 20 Ci/mol, 0.5 $\mu$g; and lane 3, [$^{14}$C]-myristoyl-ACP, 20 Ci/mol, 0.25 $\mu$g. Reaction mixtures of 100 $\mu$L containing the indicated proteins prepared and processed as described in Experimental Procedures were analyzed in the following lanes: lane 4, 25 $\mu$M [$^{14}$C]ACPSH; lane 5, 25 $\mu$M [$^{14}$C]-ACPSH + 25 $\mu$M myristoyl-ACP; lane 6, reverse reaction 1, 25 $\mu$M myristoyl-ACP + 25 $\mu$M HlyC followed by 25 $\mu$M [$^{14}$C]-ACPSH; lane 7, reverse reaction 2, 25 $\mu$M myristoyl-ACP + 50 $\mu$M HlyC followed by 25 $\mu$M [$^{14}$C]ACPSH.

addition, acyl-enzyme intermediate forms of Y150G and S20A were virtually invisible; these mutants each had greatly impaired but detectable enzyme activity. Y70G had no detectable enzyme activity and barely perceptible acyl-HlyC formation. Mutants with moderately impaired activity, R24A, S58A, and R87A varied in the amounts of acyl-HlyC formed. R24A showed no reduction in acyl-enzyme formation compared to wild type, while R87A acyl-intermediate formation was reduced. S58A probably formed about the same amount of acyl-enzyme as wild type, but most of it, in contrast to wild type, was in the form of acyl-HlyC dimer. Although wild-type acyl-HlyC showed no tendency to aggregate, several of the mutants formed acyl-HlyC dimers; this was especially pronounced with S58A. Although its band corresponding to acyl-HlyC monomer was diminished compared to wild-type acyl-HlyC formation, acyl-HlyC S58A showed a comparable formation of acyl-HlyC when band intensities of both forms, acyl-HlyC monomer and acyl-HlyC dimer, were considered. Similar behavior was seen with S76A, E67A, R87K, and Y150F.

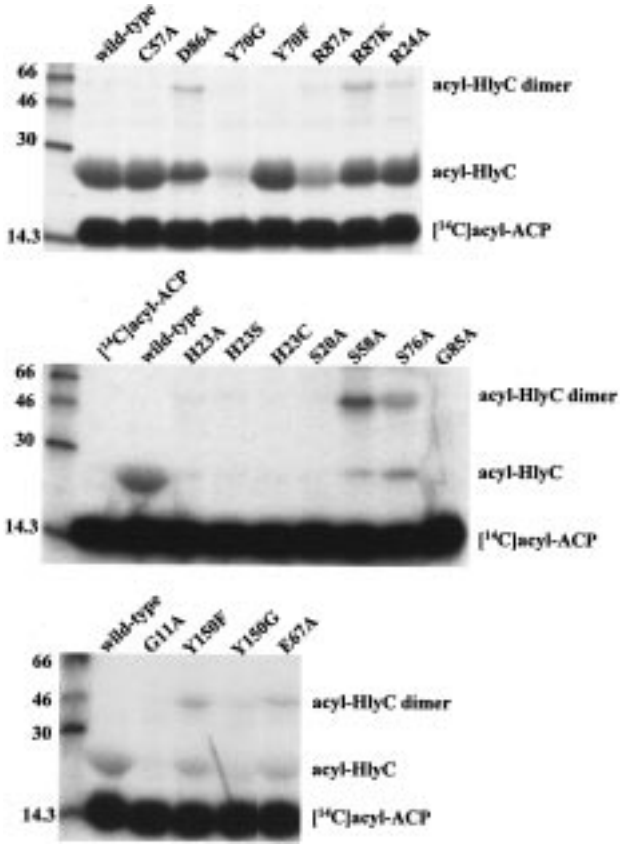


FIGURE 3: Fluorographic demonstration of [$^{14}$C]myristoyl-HlyC intermediate formation by wild-type and mutant HlyCs. All reactions except one contained 2.5 $\mu$M [$^{14}$C]myristoyl-ACP and 50 $\mu$M of the indicated wild-type or mutant $His_6$-S-tag-HlyC. The exception contained only 2.5 $\mu$M [$^{14}$C]myristoyl-ACP. A reaction containing wild-type $His_6$-S-tag-HlyC was done with each set of mutant $His_6$-S-tag-HlyC experiments to provide a reference for comparison. The indicated proteins were mixed under the previously reported assay conditions of 100 $\mu$L. After incubation for 10 min at 4 °C, proteins were precipitated with 10% trichloroacetic acid at 4 °C, collected, dissolved in 20 $\mu$L of 2% SDS sample buffer (*22*) that contained 4 M urea, and subjected to SDS−15% PAGE and fluorography as described in Experimental Procedures. Radioactive mass standards were run, and kilodalton values are shown.

According to partial reaction 1, acyl-enzyme formation would be preceded during catalysis by noncovalent binary

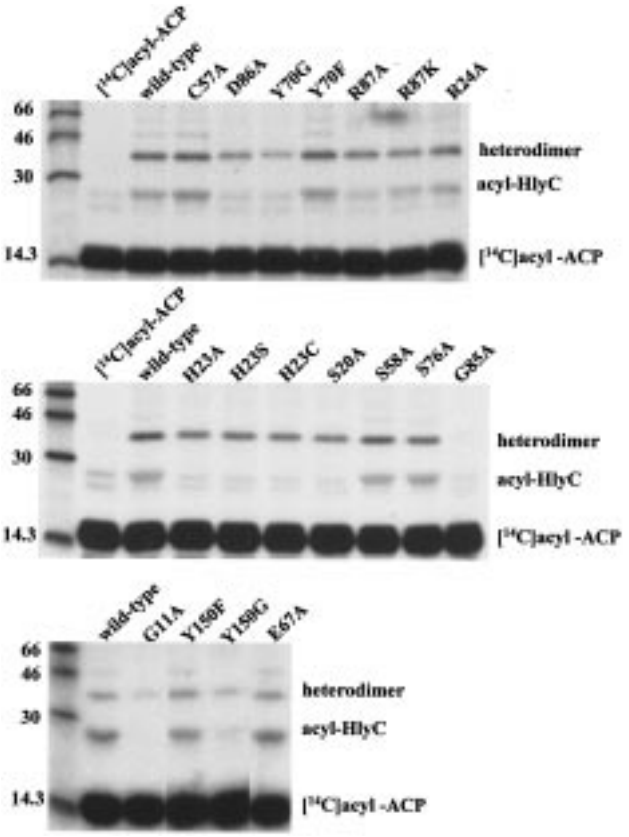


FIGURE 4: Fluorographic demonstration of myristoyl-ACP × HlyC heterodimer formed in the presence of dimethylsuberimidate. Reactions contained 2 $\mu$M [$^{14}$C]myristoyl-ACP and 4.5 $\mu$M wild-type or mutant $His_6$-S-tag-HlyC and were assembled as described previously for assay of acyltransferase activity (*16*). Dimethyl suberimidate (10 mM) was added, and after 10 min at 25 °C, reaction was halted by the addition of 100 mM ammonium acetate. A reaction containing [$^{14}$C]myristoyl-ACP only was done as a reference. The proteins were precipitated, separated, and analyzed as described in the caption for Figure 3. A reaction containing wild-type $His_6$-S-tag-HlyC was done with each set of mutant $His_6$-S-tag-HlyC experiments to provide a reference for comparison. Radioactive mass standards were run, and kilodalton values are shown.

complex formation between acyl-ACP and enzyme. An acyl-ACP × HlyC heterodimer is readily visualized upon treatment with dimethyl suberimidate (*16*). HlyC mutants impaired in catalysis and acyl-enzyme formation may, depending upon the function of the mutated residue, still be able to form heterodimer normally. Notably, three different mutations of the single histidine residue conserved among all RTX C proteins resulted in robust formation of chemically cross-linked heterodimer, acyl-ACP × HlyC, by each mutant despite complete loss of acyl-enzyme formation and activity (Figure 4 and Table 4). The faint bands evident in the lanes where the histidine mutant behaviors were analyzed stem from chemical cross-linking of acyl-ACP, whose self-cross-linking is shown. The tendency of ACP to self-associate is documented (*28*). Comparison of chemically cross-linked myristoyl-ACP ([$^{14}$C]acyl-ACP in Figure 4) and myristoyl-ACP and wild-type HlyC chemically cross-linked (wild type in Figure 4) verify faint banding from acyl-ACP multimers in the vicinity of a band stemming from acyl-HlyC. Actually, the ACP multimers sandwich the latter band. Other mutants that had no detectable enzyme activity, G11A, Y70G, and G85A, showed less or no heterodimer formation. Two mutants whose acyltransferase activity was greatly impaired showed about the same (S20A) and low (Y150G) amounts of heterodimer in Figure 4 compared to that formed by wild-type HlyC. Except for D86A and possibly Arg87, mutants that formed reduced amounts of chemically cross-linked heterodimer compared to wild type, the remaining mutants of HlyC resembled wild type in their ability to chemically cross-link with acyl-ACP.

## DISCUSSION

Definition of the complexes formed among the substrates and enzyme during the HlyC-catalyzed reaction was facilitated by their protein natures compared to enzyme reactions, where substrates are small molecules. Demonstration of partial reaction 1 reversibility ruled out the existence of a ternary complex, and as previously reported (*16*), no ternary complex of myristoyl-ACP, HlyC, and proHlyA is detectable. Binary complex formation between myristoyl-ACP and HlyC is observed upon chemical cross-linking, but it does not occur if HlyC is denatured (*16*). Furthermore, acyl-enzyme intermediate instantly disappears, forming HlyA upon the addition of proHlyA (*16*). Observations of the capacities of various HlyC mutants to form binary complexes and acyl-enzyme intermediates reinforced the plausibility that binary complex formation is on a direct route to acyl-enzyme formation, the reaction path illustrated in partial reaction 1. These findings, while not proving it, are compatible with a ping-pong kinetic mechanism. An ordered ping-pong mechanism requires that there be a transfer of a moiety from the substrate to the enzyme followed by release of the first product and the subsequent binding of the second substrate, in that order. This requirement gives rise to the two half-reactions shown above and the capacity for exchange of a label between substrate and product within a partial reaction. This property is not shared by sequential reaction mechanisms; they require all substrates and products to be present for any exchange to occur (*29*). The value of the experimental demonstration of partial reactions has been compromised in the past because of the uncertainty of contamination of a partial reaction by the subsequent reaction's substrates or products. Contamination of partial reaction 1 above with the other substrate proHlyA (or product, HlyA) was impossible since the *hlyA* gene did not exist in the cells used to express HlyC and ACPSH. Each of the proteins employed in the acyltransfer reaction was separately subcloned and expressed, rendering *in vivo* contamination impossible. The robust transfer of labeled ACPSH into the form of acyl-ACP shown in Figure 1 supports a ping-pong mechanism for HlyC *(30)*. Steady-state kinetic studies to resolve further the kinetic mechanism of the internal protein acyltransferase are in progress.

Although mutation of four different residues (Gly11, His23, Tyr70, and Gly85) to alanine or glycine resulted in total loss of acyltransferase activity, Y70G was restored to wild-type activity upon mutation to phenylalanine. Thus the tyrosine hydroxyl group was not essential for Tyr70's function in HlyC. As shown by the greatly impaired ability of G11A or G85A to form heterodimer, the glycine residues likely are important elements in HlyC structure rather than fulfilling an essential catalytic role. Mutation of His23 to cysteine or serine did not restore any detectable activity; this was the only residue that could potentially bear an acyl group whose mutation consistently led to elimination of activity.

In contrast to the complete abolition of enzyme activity of all His23 mutants, each of the His23 mutants formed an apparently normal amount of chemically cross-linked myristoyl-ACP × HlyC heterodimer compared to that formed by wild-type HlyC (Figure 4). Consequently, enzyme−substrate complex formation was not noticeably impaired by mutation of His23. Acyl-enzyme intermediate formation, which occurred after enzyme−substrate complex formation, was, however, not observed with any mutant His23 HlyC. These findings plus the previously reported observation that HlyC activity is protected by the substrate myristoyl-ACP from inhibition by diethyl pyrocarbonate, which specifically modifies histidine residues (*26*), add to the evidence that His23 has an essential role in the catalytic site of HlyC after the binding of substrate. Histidine residues are critical in many enzyme-catalyzed transfer reactions, but their catalytic role has been as acid/base catalysts rather than as nucleophiles. An exception is the acyl-imidazole intermediate formed during enzymatic catalysis in the covalent binding of complement component C4 to the surface of pathogens (*31*). An internal thioester of C4B is attacked by a histidine residue to form an acyl-imidazole intermediate, and the acyl group is subsequently transferred to amino or hydroxyl nucleophiles. This reaction is curiously analogous to proHlyA activation to HlyA, which entails proHlyA internal $\epsilon$-amino group acylation by acyl transfer from the thioester acyl-ACP via an acyl-HlyC, possibly an acyl-imidazole, to the amino nucleophile.

The impaired activity seen upon mutation of conserved Tyr150 to glycine was recovered upon mutation to phenylalanine, showing that, like Tyr70, the phenolic hydroxyl group was not essential for the role of Tyr150 in the acyltransfer reaction. These findings agree with the reported observation that *N*-acetylimidazole, a specific modifier of tyrosine hydroxyl groups, does not inhibit HlyC activity, while modification of tyrosines by nitration of the aromatic ring with tetranitromethane to form 3-nitrotyrosine inhibits acyltransferase activity (*19*). Aromatic residues have been shown to function biologically in some instances to stabilize cations through their interaction with the $\pi$ electrons of aromatic rings (*32*). Such a role in HlyC catalysis is, however, not apparent. The residue substitutions that support activity may reflect the requirement for a bulky, hydrophobic residue. Perhaps such is needed to ensure an optimal environment for the acyl group that is transferred from acyl-ACP to form the acyl-enzyme intermediate. The inhibition of HlyC by tetranitromethane cited above is prevented if myristoyl-ACP is bound to HlyC suggesting that tyrosine(s) crucial to activity modified by the reagent is (are) in the active-site vicinity (*19*). This view is supported by the observation that both single-site tyrosine mutations to glycine showed impaired formation of chemically cross-linked myristoyl-ACP × HlyC heterodimer. This finding suggests a function for the two conserved tyrosine residues in enzyme/substrate binding prior to catalysis of partial reaction 1. Possibly one or both of the single-site tyrosine modifications led to a change in HlyC that hindered myristoyl-ACP access to the active site. Aromatic residues have been shown to be located in fatty acyl binding regions of proteins that bind acyl groups while exercising their biological roles (*33*−*35*). For example, a particular tryptophan residue is intimately associated with the acyl group during catalysis of a lux-specific acyl-ACP thioesterase that forms an acyl-enzyme intermediate (*36*). The substrate protection of tyrosine from chemical modification cited above does suggest that tyrosine is important in the vicinity of the active site of HlyC.

S20A was markedly impaired kinetically with a greatly reduced $V_{max}^{app}$, but it formed myristoyl-ACP × HlyC heterodimer like wild-type enzyme. Thus enzyme−substrate complex formation was unimpaired. Acyl-enzyme intermediate was, however, not detectable even though the mutant had minimal rates of catalysis. Ser20 appears to be very important in HlyC catalysis, but its function is not evident. It is not likely to be the covalent catalytic residue because its mutation to an alanine residue, which would be incapable of such a function, still permits a low amount of enzyme activity to occur.

R24A mutant, which was slightly impaired kinetically through a change in $V_{max}$, was restored to wild-type activity upon mutation to another residue capable of bearing a positive charge, R24K *(19)*. R24A enzyme appeared to have heterodimer and acyl-enzyme formation like that of wild-type enzyme. A structural role for this residue cannot be ruled out, but R24A, in contrast to R87A, did not recover full activity upon slow refolding compared to fast renaturation (*19*). Possibly Arg 24 has a specific as yet unknown function in partial reaction 2 of HlyC catalysis.

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