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†

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ABSTRACT: HlyC is an internal protein acyltransferase that activates hemolysin, a toxic protein produced by pathogenic *Escherichia coli*. Acyl−acyl carrier protein (ACP) is the essential acyl donor. Separately subcloned, expressed, and purified prohemolysin A (proHlyA), HlyC, and [1-^{14}C]myristoyl-ACP have been used to study the conversion of proHlyA to HlyA [Trent, M. S., Worsham, L. M., and Ernst-Fonberg, M. L. (1998) *Biochemistry 37*, 4644−4655]. HlyC and hemolysin belong to a family of at least 13 toxins produced by Gram-negative bacteria. The homologous acyltransferases of the family show a number of conserved residues that are possible candidates for participation in acyl transfer. Specific chemical reagents and site-directed mutagenesis showed that neither the single conserved cysteine nor the three conserved serine residues were required for enzyme activity. Treatment with the reversible histidine-modifying diethyl pyrocarbonate (DEPC) inhibited acyltransferase activity, and acyltransferase activity was restored following hydroxylamine treatment. The substrate myristoyl-ACP protected HlyC from DEPC inhibition. These findings and spectral absorbance changes suggested that histidine, particularly a histidine proximal to the substrate binding site, was essential for enzyme activity. Site-directed mutageneses of the single conserved histidine residue, His23, to alanine, cysteine, or serine resulted in each instance in complete inactivation of the enzyme.

Pathogenic *Escherichia coli* secrete a toxic protein, hemolysin (HlyA),[1] which binds to and lyses mammalian cell membranes and, at lower concentrations, perturbs cell signal transduction and release of inflammatory mediators (*1, 2*). It is one of a family of homologous membrane-active toxins, RTX (repeats in toxin) toxins, of similar mechanism produced by Gram-negative bacteria (*3*−*5*). The toxin arises from the expression of the *hlyCABD* gene cluster. The *hlyA* gene product is nontoxic prohemolysin (proHlyA) which is converted intracellularly to mature toxin, HlyA, by the action of the *hlyC* gene product, HlyC (*6*). HlyC, an internal protein acyltransferase, catalyzes the transfer of a fatty-acyl group from acyl-ACP to α-amino groups of lysine residues 564 and 690 of proHlyA, converting it to the toxin, HlyA, which is secreted (*7*−*9*).

Internal fatty acylation of proteins is a recognized means of modifying the biological behavior of a protein, enabling regulatory mechanisms such as signal transduction, and many toxins exert their effects through distortion of cellular signaling mechanisms. Numerous instances of protein internal fatty acylation, generally via thiol esterification of cysteine residues, have been reported. The acyltransferases catalyzing these reactions have been elusive. Recently, however, two protein−palmitoyl acyltransferases have been isolated (*10, 11*). Although several instances of posttranslational protein modification by internal fatty acylation through amide linkage have been reported (*12* and references therein, *13*−*15*), the enzymes have been neither isolated nor characterized. Recently, we reported the separate subcloning and expression of each of the proteins involved in the internal acylation of proHlyA to form toxic HlyA (*8*). The availability of purified, substrate quantities of each protein participating in the reaction enabled an extensive characterization of the reaction and of purified HlyC, a unique acyltransferase. Notably, a reactive acyl-HlyC intermediate is formed (*8*). Comparison of the deduced sequences of C proteins encoded by 13 different RTX operons showed a high degree of homology (*16*). Among the conserved residues were several residues known to be catalytically important in other acyl transfer or related reactions.

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[1] Abbreviations: proHlyA, hemolysin A protoxin; HlyA, hemolysin A toxin; RTX, repeats in toxin; HlyC, acyl−acyl carrier protein: prohemolysin A 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; MES, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; NEM, *N*-ethylmaleimide: PCMB, *p*-chloromercuribenzoate; PMSF, phenylmethanesulfonyl fluoride; AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride; and DEPC, diethyl pyrocarbonate.

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Table 1: Primers Used To Construct Recombinant DNA and HlyC Mutants[a]

primer	sequence (5′ → 3′)
HlyC1	ATGAATATAAACAAACCATAGAGATTCTT
HlyC2	TTAACCAGTTAATGAAAAATTAAAATCTGA
S20A upper	CCTGGCTATGGGCCAGTGCTCCACTACACAGAAACTGGCC
S20A lower	GGCCAGTTTCTGTGTAGTGGAGCACTGGCCCATAGCCAGG
H23A upper	GGCTATGGGCCAGTTCTCCACTAGCTAGAAACTGGCCAGTATC
H23A lower	GATACTGGCCAGTTTCTAGCTAGTGGAGAACTGGCCCATAGCC
H23C upper	GGCTATGGGCCAGTTCTCCACTATGTAGAAACTGGCCAGTATC
H23C lower	GATACTGGCCAGTTTCTACATAGTGGAGAACTGGCCCATAGCC
H23S upper	GGCTATGGGCCAGTTCTCCACTATCTAGAAACTGGCCAGTATC
H23S lower	GATACTGGCCAGTTTCTAGATAGTGGAGAACTGGCCCATAGCC
C57A upper	GATTACCCTGTCGCGTATGCTAGTTGGGCTAATTTAAGTTTAG
C57A lower	CTAAACTTAAATTAGCCCAACTAGCATACGCGACAGGGTAATC
S58A upper	GATTACCCTGTCGCGTATTGTGCTTGGGCTAATTTAAGTTTAG
S58A lower	CTAAACTTAAATTAGCCCAAGCACAATACGCGACAGGGTAATC
S76A upper	CTTAATGATGTTACCGCTTTAGTTGCAGAAGACTGGACT TCAGG
S76A lower	CCTGAAGTCCAGTCTTCTGCAACTAAAGCGGTAACATCATTAAG

[a] Mutant amino acid codons are underlined.

The chemical mechanism of catalysis is not known for any internal protein acyltransferase. The conservation of particular residues among 13 different internal protein acyltransferases provided an opportunity to identify residues that may be important in the enzymatic activity of RTX C proteins. In addition, the demonstration of an acyl-enzyme intermediate in the HlyC-catalyzed reaction provided further clues as to possible catalytic residues since at least one amino acid must be included that could bear an acyl group. By chemical modifications and site-directed mutations, we have identified residues among a portion of those conserved that may be required for optimum enzyme function.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Myristate was from New England Nuclear. Sigma was the source of *N*-ethylmaleimide (NEM), *p*-chloromercuribenzoate (PCMB), phenylmethanesulfonyl fluoride (PMSF), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), diethyl pyrocarbonate (DEPC), and Kodak film for fluorography. *Eco*RV, *Dpn*I, and Deep Vent DNA polymerase were from New England Biolabs. *Pfu*Turbo DNA polymerase was from Stratagene. All chemicals were reagent-grade. Novagen was the source of S-protein alkaline phosphatase conjugate and His-bind resin. Urea-containing buffers were always freshly prepared.

Bacterial Strains, Media, and DNA Manipulations. E. coli strains were BL21(DE3)pLysS and NovaBlue from Novagen and X-L2 Blue from Stratagene. Cells were grown in Luria broth except for expression of 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 subcloning of the native *hlyC* gene or site-directed mutagenesis of *hlyC* were from Integrated DNA Technologies and are described in Table 1. Mutation of DNA was confirmed by DNA sequence analysis (*17*) at the Molecular Genetics Facility at the University of Georgia.

HlyC was expressed as a His_6−S-tag fusion protein from pTXC2, which was constructed by blunt-end ligation of the polymerase chain reaction (PCR) product of the *hlyC* gene template from pHly152 (*18*) into the *Eco*RV restriction endonuclease site of pET30a(+) (Novagen). The primers, HlyC1 and HlyC2, that encompassed the *hlyC* gene are shown in Table 1. The His_6−S-tag−HlyC subclone expressed and exhibited specific activity and kinetic parameters similar to those of the S-tag−HlyC fusion protein that has been extensively characterized (*8*). Fusion protein was purified to homogeneity by Ni^{2+} ligand chromatography as described below.

Site-Directed Mutagenesis. The round circle PCR method described in the Quik-Change site-directed mutagenesis kit protocol (Stratagene) generated site-directed mutations in *hlyC* with the plasmid pTXC2 as the reaction template. The rationale involved whole-plasmid PCR amplification using 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. ProHlyA, ACPSH, and radiolabeled myristoyl-ACP were obtained as described by Trent et al. (*8*). Myristoyl-ACP was purified and evaluated as described (*19*) and stored in aliquots at −80 °C. The fusion protein N-terminal S-tag−HlyC was expressed and purified as previously described (*8*); it was used for the chemical modification studies presented herein. Another HlyC fusion protein, an N-terminal His_6−S-tag whose construction and expression is described above, was employed for site-directed mutagenesis. N-Terminal His_6−S-tag−HlyC was extracted from inclusion bodies obtained from a 500 mL cell culture prepared as described by Trent et al. (*8*) and purified with an affinity His-Bind Ni^{2+} chelation resin. Fusion protein (15 mg) in 7.5 mL of 20 mM Tris (pH 7.9), 0.5 M NaCl, 5 mM imidazole, and 6 M urea was applied to 1.5 mL of His-Bind resin. The column was washed with the same buffer followed by a 20 mM Tris (pH 7.9), 0.5 M NaCl, 20 mM imidazole, and 6 M urea wash. Fusion protein was eluted with 20 mM Tris (pH 7.9), 0.5 M NaCl, 500 mM imidazole, and 6 M urea. Urea was removed by dialysis for 3 h against 25 mM HEPES (pH 8.0), 5 mM EDTA, and 2 mM DTT that contained 3 M urea, followed by 3 h against the same buffer except that it contained 1 M urea. The final dialysis was for 16 h against buffer with no urea. After clarification of the protein solution by centrifugation at 30000*g*, fusion protein

was precipitated overnight at 65% $(NH_4)_2SO_4$ saturation, collected by centrifugation at 30000*g*, and dissolved in 25 mM HEPES (pH 8.0), 5 mM EDTA, and 2 mM DTT. The yield was ~4 mg.

Assays. Protein was measured as described by Bradford (*20*). The purity of each protein used was assessed by SDS−PAGE (*21*). The HlyC-catalyzed transfer of radiolabeled acyl groups from acyl-ACP to proHlyA to form radiolabeled HlyA was measured as described previously (*8*).

Chemical Modification of Selected HlyC Residues. A solution of NEM was prepared in water and adjusted to 260 mM [$A_{302\ nm}$ 620 M^{-1} cm^{-1} (*22*)]. S-tag−HlyC, 2.5 mg in 1 mL 0.1 M KPO_4 (pH 7.0), was exposed to 0.1 or 1 mM NEM buffer at 25 °C for 15 min and then replaced into 25 mM HEPES (pH 8.0) and 0.1 M NaCl with Sephadex G-25 chromatography. Upon elution, the acyltransferase activity of the protein was measured. A control aliquot of S-tag−HlyC was identically processed except that it was not exposed to NEM. Control and treated enzyme (10 μg each) were assayed.

A 10 mM solution of PCMB [$A_{233nm} = 1.69 \times 10^4$ M^{-1} cm^{-1} (*22*)] in 0.01 M KPO_4 (pH 7.0) was used to make 0.1 and 1 mM solutions containing 210 μg of S-tag−HlyC in 20 mM MES (pH 6.0). After 10 min at 25 °C, reagent was removed by use of a Centricon (Millipore) with a 5000 molecular weight limit. A 300 μL wash of HEPES (pH 8.0), 5 mM ETDA, and 1 mM DTT was passed through the membrane, and the protein was put into 100 μL HEPES (pH 8.0), 5 mM ETDA, and 1 mM DTT and assayed immediately for acyltransferase activity. A control aliquot of S-tag−HlyC was identically treated except that it was not exposed to PCMB.

Solutions (10 mM) of PMSF and AEBSF were prepared in 2-propanol and dimethyl sulfoxide, respectively. S-tag−HlyC, 50 μg in 25 mM Hepes (pH 8.0), was exposed to 0.1 and 1 mM PMSF or AEBSF or only their respective solvents (controls) for 10 min at 25 °C, after which the reagent was removed by Centricon filtration as described above. Acyltransferase activity was assayed.

A 65 mM solution of DEPC in acetonitrile was made. Its concentration was measured by measuring the A_{240nm} upon reaction with 10 mM imidazole in 0.1 M KPO_4 (pH 6.0) [A_{242} of *N*-carbethoxyhistidine = 3200 M^{-1} cm^{-1} (*23*)]. S-tag−HlyC was precipitated at 65% saturation with $(NH_4)_2SO_4$ and brought up (~2 μg/μL) in 0.1 M KPO_4 (either pH 6.0 or 8.0), and 22 μL aliquots were exposed to 1 μL portions of acetonitrile containing none (control) or the desired amount of DEPC. After 10 min at 25 °C, the reactions were quenched with 5 μL of 50 mM imidazole, and acyltransferase activity was measured. Also, the time−response of DEPC effect on S-tag−HlyC at 25 °C was measured by using a larger reaction solution and removing aliquots for quenching and assay at intervals. The effects of hydroxylamine on S-tag−HlyC treated with 2 mM DEPC and quenched with imidazole was examined by adding NH_2OH (pH 7.0) to 100 mM. Aliquots were removed at intervals and assayed for acyltransferase activity. Controls were HlyC exposed in parallel to acetonitrile, imidazole, and/or hydroxylamine.

Finally, either 2 μM myristoyl-ACP or ACPSH was added to 2 mM DEPC reactions with S-tag−HlyC. The ACPs were also added to the acetonitrile controls and were removed from both reactions (after DEPC reaction) and controls by precipitation of HlyC at 65% $(NH_4)_2SO_4$ at neutrality prior to measurement of acyltransferase activity. ACP and its derivatives do not precipitate under these conditions. [^{14}C]-Myristoyl-ACP was, of course, newly added for measurement of enzyme activity.

Spectral Measurements. Reaction of S-tag−HlyC with DEPC was monitored spectrally with a Hewlett-Packard 8452A diode-array spectrophotometer at wavelengths ranging from 200 to 500 nm at 2 nm increments. A cuvette partitioned to $^3/_4$ its height with a quartz wall was used with the enzyme solution [1.0 mg/mL S-tag−HlyC in 0.1 M KPO_4 (pH 6.0)] placed in one sector and sufficient DEPC for a final concentration of 1 mM placed in the other. A spectrum taken prior to mixing served as the blank. Following mixing of the two solutions, spectra were taken at intervals.

RESULTS

Residues Essential for Acyltransferase Activity. Comparison of the deduced amino acid sequences of C proteins from 13 different RTX toxins revealed extensive homology (Figure 1). Residues 10−80 of HlyC contain 22 residues that are identical in all RTX toxins, and 11 more residues are conserved in type of residue. An acyl-enzyme intermediate has been demonstrated for HlyC (*8*), and residues with acylation potential are among those conserved in the diverse C proteins compared above. Most of these are shown in Figure 1, where identical residues are shaded. For example, HlyC possesses a single cysteine, Cys57, and it is conserved among all RTX C proteins. The sulfhydryl-reactive agents NEM and PCMB, however, had no effect on S-tag−HlyC activity (Figure 2). Similarly, three serine residues (Ser20, Ser58, and Ser76 in HlyC) are conserved in all 13 C proteins, but reagents that would be expected to react with a reactive serine residue, PMSF and AEBSF, had no effect on acyltransferase activity (Figure 2). Another residue that has been shown to participate in the catalytic mechanism of many enzymatic acyl group transfers is histidine, although its role has been as an acid−base catalyst rather than as a nucleophile. Among the RTX C proteins, a single histidine is conserved, His23 in HlyC. DEPC is the most suitable reagent for specific modification of histidines. Preferring unprotonated nucleophiles, it reacts with high specificity with protein histidine residues at pH 6.0, while at pH 8.0 the reagent may modify tyrosine, cysteine, and lysine residues (*23*). Exposure of S-tag−HlyC to DEPC at pH 6 resulted in loss of 84% of acyltransferase activity compared to parallel assays where HlyC had not been treated with DEPC but exposed to solvent (Figure 2). In contrast, about 70% of acyltransferase activity was retained upon treatment of S-tag−HlyC with DEPC at pH 8 (Figure 2). Results following exposure of S-tag−HlyC to DEPC at pH 8.0 confirmed that the cysteine residue was not essential for HlyC activity, and suggested that neither ϵ and α-amino groups nor tyrosine residue hydroxyl groups were necessary for HlyC catalysis. The inhibition of HlyC activity by modification of histidine residue(s) was investigated further.

Histidine Modification. Inhibition of S-tag−HlyC by DEPC was dependent upon the concentration of the inhibitor (Figure 3). A 10 min incubation of S-tag−HlyC with increasing concentrations of DEPC at pH 6.0 caused linearly

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                  20        30        40        50        60        70        80
HlyC        GHVSWLWASS PLHRNWPVSL FAINVLPAIQ ANQYVLLTRD DYPVAYCSWA NLSLENEIKY LNDVTSLVAE
HlyC1       GHVSWLWASS PLHRNWPVSL FAINVLPAIR ANQYALLTRD NYPVAYCSWA NLSLENEIKY LNDVTSLVAE
HlyC2       GHVSWLWASS PLHRNWPVSL FAINVLPAIR ANQYVLLTRD NYPVAYCSWA NLSLENEIKY LNDVTSLVAE
EhxC        GKVAWLWACS PLHKKWPLSV FAINVIPAIQ TNQFALLIKD ELPVAFCSWA SLDLECEVKY INDVTSLYPK
ApxIC       GEVAWLWASS PLHRKWPLSL LAINVLPAIE SNQYVLLKRD GFPIAFCSWA NLNLENEIKY LDDVASLVAD
ApxIIC      GQIAWLWANS PMHRNWSVSL LMKNVIPAIE NDQYLLLVDD GFPIAYCSWA KLTLESEARY VKDTNSLKID
ApxIIIC     GHIAWLWANS PLHKEWSISL FTKNILPAIQ HDQYILLMRD EFPVAFCSWA NLTLTNEVKY VRDVTSLTFE
AaltC       GYVAWLWANS PLHRNWSLSL LAINVLPAIQ YGQYTLLMRD GVPIAFCSWA NLSLENEIKY LEDVSSLVYD
LktC(A1)    GNITWLWMNS SLHKEWSCEL LARNVIPAIE NEQYMLLIDN GIPIAYCSWA DLNLETEVKY IKDINSLTPE
LktC(T3)    GNITWLWMNS SLHKEWSCKL LACNVIPAIE NEQYMLLVDN GIPIAYCSWA DLNLETEVKY IKDISSLTSD
LktC(A11)   GNITWLWMNS PLHKEWSCEL LARNVIPAIE NEQYMLLIDD GIPVAYCSWA DLNLENEVKY IKDISSLTLE
PlktC       GNITWLWMNS PLHREWSCEL LARNVIPAIE NQQYMLLIDN DVPIAYCSWA DLSLETEVKY IKDISSLTPE
CyaC        GNIAWLWMNS PMHRDWPVHL LARNTLAPIQ NEQYMLLIDD DVPVAYCSWA LMDADTELSY VMAPSSLGGN
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FIGURE 1: Alignment of the partial amino acid sequences of 13 RTX toxin C gene products. Each of the RTX toxin C proteins is described as follows: HlyC, *E. coli* HlyC, pHly152-encoded, accession no. P06736; HlyC1, *E. coli* strain J96 HlyC, chromosome-encoded, accession no. P09984; HlyC2, *E. coli* strain 2001 HlyC, chromosome-encoded, accession no. p09985; EhxC, *E. coli* 0157:H7 strain EDL 933 EhxC, plasmid-encoded, translation of nucleotide sequence accession no. X80891; ApxIC, *Actinobacillus pleuropneumoniae* Apx-IC, accession no. P55132; ApxIIC, *A. pleuropneumoniae* Apx-IIC, accession no. P15376; ApxIIIC, *A. pleuropneumoniae*, Apx-IIIC, accession no. Q04474; AaltC, *Actinobacillus actinomycetemcomitans* LktC, accession no. P16461; LktC(A1), *Pasteurella haemolytica* serotype A1 LktC, accession no. P16533; LktC(T3), *P. haemolytica* serotype T3 LktC, accession no. P55120; LktC(A11), *P. haemolytica* serotype A11 LktC, accession no. p55121; PlktC, *P. haemolytica*-like sp. LktC, accession no. p55124; CyaC, *Bordetella 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 alignment was done with the Hibio Prosis protein analysis software by Hitachi. Amino acid sequence numbers refer to positions in the *E. coli* HlyC encoded by plasmid pHly152.

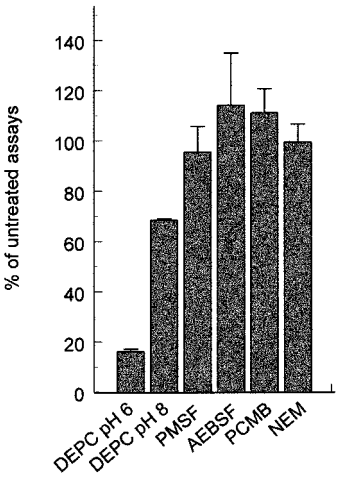

FIGURE 2: Effects of potential inhibitors on HlyC activity. The methods of exposing S-tag−HlyC to each of the various inhibitors and neutralization of each of the inhibitors prior to measurement of acyltransferase activity are provided in Experimental Procedures. The control assays employed S-tag−HlyC that was exposed to the respective solvents and procedures but not reagents. The pH 8.0 controls assay had an activity of 35.3 ±1.1 nmol of acyl group transferred (average ± standard deviation). Assays were done as previously described (*8*). Control assays done at pH 6.0 for DEPC inhibition had an activity of 23.0 ± 1.8 nmol of acyl group transferred. All inhibitor concentrations were 1 mM, and the bars represent experiments that contained the indicated inhibitors.

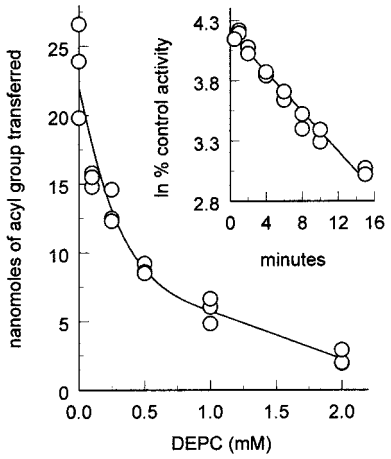

FIGURE 3: DEPC effects on acyltransferase activity at pH 6.0. Preparation of S-tag−HlyC and treatment with different concentrations of DEPC was done as described in Experimental Procedures. Acyltransferase activity was assayed as previously described (*8*). The data points are the results of individual assays. The line is a weighted least-squares plot described by the data. The inset shows the natural log of the percentage of the control activity relative to the time of exposure to 1 mM DEPC. To observe activity over time, a solution of S-tag−HlyC, 200 μg in 500 μL 0.1 M KPO_4 (pH 6.0), was prepared, and two 25 μL aliquots were placed in assay tubes containing 5 μL of 50 mM imidazole (for reaction quenching). These were 0 time acyltransferase assay controls. The remainder of the enzyme solution was treated with 1 mM DEPC, and 25 μL aliquots were removed at the indicated times for quenching and enzyme assay. Enzyme activity from individual assays is shown as ln % of control activity (○) which was 37.7 nmol of acyl group transferred. The line is a linear regression plot with a correlation coefficient of 0.97.

increasing losses of HlyC activity up to 0.5−1 mM, at which point 20% of the original activity remained. The curve was biphasic, and by 2 mM inhibitor, only 7% of HlyC activity remained. Carbethoxyhistidine-modified proteins show distinctive absorbance spectra compared to those of unmodified proteins (*23*). That histidine was the target of DEPC was confirmed by the appearance of a new absorbance peak at 242 nm, characteristic of carbethoxyhistidine. There was no decrease in A_{278nm}, which would have occurred had tyrosine been modified (*24, 25*) (spectra not shown). Inactivation of S-tag−HlyC by DEPC followed first-order kinetics (Figure 3, inset), and the loss of acyltransferase activity with time was inversely proportional to the formation of carbethoxyhistidine shown by the increase in A_{242nm} (Figure 4).

Hydroxylamine removes the carbethoxy groups from modified histidyl and tyrosyl residues (*23, 26*), and spectral evidence presented above indicated that only histidine residues were modified. Treatment of DEPC-inactivated S-tag−HlyC with 100 mM hydroxylamine showed an increase in enzyme activity with time, leveling off after ~200

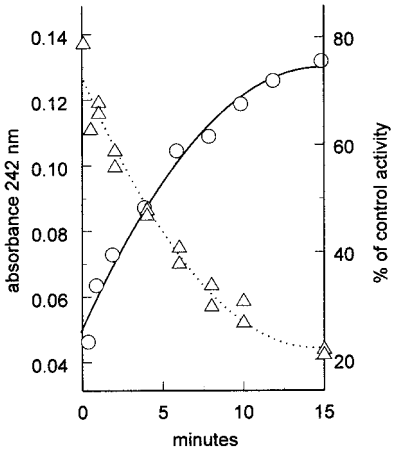

FIGURE 4: Relationship between acyltransferase activity decrement and histidine modification upon exposure of HlyC to DEPC. To observe activity over time, a solution of S-tag−HlyC, 200 μg in 500 μL of 0.1 M KPO_4 (pH 6.0), was prepared, and two 25 μL aliquots were placed in assay tubes containing 5 μL of 50 mM imidazole (for reaction quenching). These were 0 time acyltransferase assay controls. The remainder of the enzyme solution was treated with 1 mM DEPC, and 25 μL aliquots were removed at the indicated times for quenching and enzyme assay. Acyltransferase was assayed as previously described (Trent et al., 1998). Enzyme activity is presented on the right axis as percent of control activity (Δ), which was 37.7 nmol of acyl group transferred. Details of the spectrophotometric experiments are given in Experimental Procedures; A_{242nm} readings are shown as $\circ$ with the scale on the left axis.

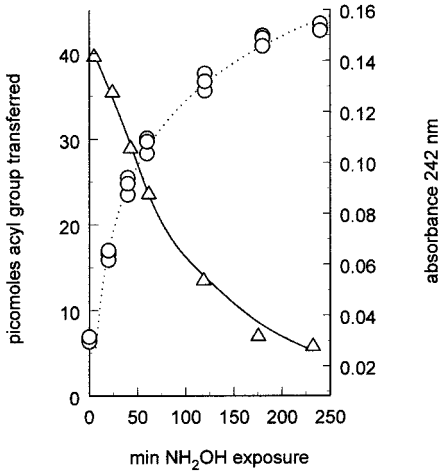

FIGURE 5: Hydroxylamine reversal of DEPC modification of HlyC and activity inhibition. S-tag−HlyC, 340 μg in 200 μL of 0.l M KPO_4 (pH 6.0), was treated with 2 mM DEPC for 10 min at 25 °C; after which the reaction was quenched with 5 mM imidazole. A control was similarly treated except that DEPC was omitted. Acyltransferase activities of 10 μg portions of enzyme were measured for control values at 0 time. After withdrawal of 0 time enzyme aliquots, the quenched reaction solution was made 100 mM in NH_2OH with freshly prepared 2 M NH_2OH (pH 7.0), and aliquots were taken and assayed at the indicated times for acyltransferase activity ($\circ$) as previously described (*8*). An identical experiment was prepared, and following the DEPC treatment, the increase in absorbance at 242 nm was measured relative to untreated ezyme; this was the 0 time reading. Following the addition of NH_2OH, the change in absorbance relative to untreated enzyme was recorded at timed intervals (Δ).

min of treatment and recovering 87% of enzyme activity (Figure 5). The recovery of enzyme activity roughly matched the decline of absorbance at 242 nm (Figure 5), which indicated the removal by hydroxylamine of the carbethoxy groups from the modified histidine residue(s). The hydroxylamine-induced recovery of enzyme activity confirmed that neither cysteine nor lysine residue modifications were responsible for the marked decline in HlyC activity upon exposure to DEPC. DEPC modification of cysteine or lysine residues is not reversed by hydroxylamine treatment (*23*). By use of the molar absorption coefficient of *N*-carbethoxyhistidine given in Experimental Procedures, DEPC modification at pH 6.0 formed about 1.3 mol of *N*-carbethoxyhistidine/mol of S-tag−HlyC. Thus the structural integrity of at least one of the six histidine residues of HlyC was essential for function.

Table 2: Effects of ACPSH or Myristoyl-ACP on DEPC Inhibition of HlyC[a]

DEPC (mM)	ACPSH present during DEPC exposure (μM)	myristoyl-ACP present during DEPC exposure (μM)	acyl group transferred[b] (pmol)
0	0	0	33.9 ± 0.1
2	0	0	6.1 ± 1.1
2	0	2	27.5 ± 2.0
2	2	0	6.4 ± 2.2

[a] The details of assembling the assays are given under Experimental Procedures. The assays were done as previously described (*8*). [b] Each determination was done at least four times, and the numbers are the averages ± the standard deviation per 5 mg of S-tag−HlyC per 5 min assay.

Exposure of S-tag−HlyC to DEPC in the presence of myristoyl-ACP, the substrate predicted by the kinetic mechanism[2] to bind first, resulted in substantial protection from DEPC modification (Table 2). Under conditions where DEPC treatment reduced S-tag−HlyC activity to 20% of the activity of unmodified enzyme, the presence of myristoyl-ACP during exposure to inhibitor protected the enzyme from the inhibitor to the extent that 81% of activity was retained. The formation of *N*-carbethoxyhistidine (measured spectrally) was also much reduced by the presence of substrate during DEPC modification of S-tag−HlyC (not shown). In contrast to the protection of the enzyme by acyl-ACP, S-tag−HlyC treated with DEPC in the presence of product, ACPSH, was not protected from inhibition.

Expression and Purification of His_6−S-tag−HlyC. To enable single-step purification of small preparations of many different HlyC mutants, a His_6−S-tag−HlyC fusion protein was prepared, expressed, and purified as described in Experimental Procedures. The fusion protein, following urea extraction from inclusion proteins (Figure 6, lane 2), was purified by chromatography on a Ni^{2+} chelation resin. Extraneous protein separated (lanes 3 and 4) following adsorption of the His_6−S-tag−HlyC to the affinity column; the bound His_6−S-tag−HlyC was eluted with 0.5 M imidazole in urea buffer (lane 5). Following renaturation by dialysis to remove urea, the acyltransferase activity of the N-terminal His_6−S-tag−HlyC was identical to that of the slightly smaller fusion protein S-tag−HlyC reported earlier and used above (*8*).

Mutation of HlyC Conserved Histidine, Cysteine, and Serine Residues. The following site-directed mutations of His_6−S-tag−HlyC were confirmed by DNA sequence analy-

[2] M. S. Trent, L. M. Worsham, and M. L. Ernst-Fonberg, (1998) manuscript in preparation.

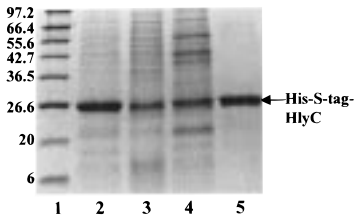

FIGURE 6: SDS−PAGE analysis of the purification of His_6−S-tag−HlyC. His_6−S-tag−HlyC was subcloned, expressed, purified, and analyzed by SDS−15% PAGE as described in Experimental Procedures. Protein from the following sources was applied in 10 μg aliquots: lane 1, mass standards (kilodaltons); lane 2, washed inclusion bodies; lane 3, Ni^{2+} chelation resin flowthrough, lane 4, Ni^{2+} chelation resin 20 mM imidazole wash; lane 5, Ni^{2+} chelation resin 500 mM imidazole eluate.

Table 3: Acyltransferase Activities of HlyC Single-Site Mutants[a]

	% of wild-type acyltransferase activity	
mutant	quick dilution	slowly refolded
H23A	2.7 ± 0.1	4.4 ± 0.1
H23S	6.6 ± 0.4	5.6 ± 0.6
H23C	11 ± 1.8	3.5 ± 1.3
C57A	90 ± 0.7	96 ± 3.5
S20A	36 ± 3.8	31 ± 0.7
S58A	78 ± 0.4	76 ± 2.1
S76A	61 ± 2.8	94 ± 5.7

[a] Acyltransferase activity was measured as previously described (*8*). 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 4−8 assays ± the standard deviation.

sis: H23A, H23C, H23S, C57A, S20A, S58A, and S76A. The mutant HlyCs expressed and purified like the wild-type N_terminal His_6−S-tag− HlyC. The effects of single, specific mutations on acyltransferase activity are shown in Table 3. Roughly 0−10% of wild-type activity was not distinguishable from zero activity, a situation that is not unusual with site-directed mutagenesis of enzymes (*27*). The mutation C57A had no effect on enzyme activity although the cysteine residue is conserved among all known RTX C proteins. Mutation of the three conserved serine residues had variable effects on acyltransferase activity. His_6−S-tag−HlyC S20A resulted in a less efficient enzyme where acyltransferase activity was ~30% that of wild-type enzyme. S58A did not affect HlyC activity. In contrast, S76A decreased acyltransferase activity to about 60% that of wild-type His_6−S-tag−HlyC when it was subjected to renaturation by quick dilution following extraction from inclusion bodies into 5 M urea buffer. When, however, S76A was slowly renatured by incremental dialysis over 22 h, its activity was like that of wild-type enzyme.

In contrast to the other conserved residue mutations, mutation of His23 appeared to destroy enzyme activity. H23A, H23S, and H23C in Table 3 show that there is essentially no detectable activity in any of the three mutations of His23. Thiol and hydroxyl groups of cysteine and serine, respectively, were unable to provide viable substitutes for the imidazole ring of His23, if that be the structurally significant portion of His23. Acyltransferase activity was routinely assayed at 4 °C; assay of the inactive His23 mutants at room temperature, even for longer periods of time, did not yield any evidence of activity.

DISCUSSION

A combination of chemical modification and site-directed mutagenesis experiments of selected conserved residues have shown that a histidine residue or residues is required for HlyC acyltransferase activity. A histidine residue may be essential in one of three ways: it can be a catalytic residue, it can be involved in substrate binding, or it can be an important structural element.

The effects of modification of HlyC by reagents reactive with particular groups suggested that no reactive serine residues, cysteine residues, or amine groups were required for acyltransferase activity. In contrast, the behavior of the enzyme upon treatment with DEPC at pH 6.0 showed that the integrity of histidine residue(s) was essential for activity. The spectral data corroborated the modification of histidine by DEPC at pH 6.0 and ruled out tyrosine modification. The almost full recovery of enzymatic function upon treatment of the DEPC-inhibited enzyme with hydroxylamine verified the requirement of histidine(s) for activity (*26*). The failure to regain 100% activity upon hydroxylamine treatment may indicate disubstitution of the histidine ring by this reagent, with subsequent ring cleavage during reversal with hydroxylamine (*23*). When HlyC was treated with DEPC in the presence of substrate myristoyl-ACP, histidine(s) responsible for the loss of activity were protected from the reagent and 81% of acyltransferase activity was preserved. If instead the reaction product ACPSH was present when HlyC was treated with DEPC, there was practically no protection from inhibition by the reagent. The uni- uni iso uni-uni ping-pong kinetic mechanism[2] of the HlyC-catalyzed reaction predicted an isomerization of the enzyme, and consequently acyl-ACP and ACPSH bind to different forms of HlyC. Protection by substrate to this extent suggests the involvement of the modified residue(s) in or proximal to the catalytic site.

Individual mutagenesis of evolutionarily conserved residues of HlyC corroborated and focused the chemical modification studies. The only residue whose mutation demonstrated that it was essential for activity was His23, one of six histidine residues in HlyC but the only histidine residue conserved among all RTX C proteins. When Ala, Cys, or Ser was substituted for His23, there was no detectable acyltransferase activity.

The single conserved cysteine and three conserved serine residues were not crucial for enzyme activity. Except for Ser58, however, mutation of the other two conserved serine residues, while not eliminating activity, adversely affected acyltransferase activity. The decreased activity observed for quickly refolded S76A compared to wild-type enzyme similarly treated suggests a role for Ser76 in folding or stabilizing the conformation of the enzyme. Upon slow refolding of denatured S76A HlyC, activity equal to wild-type enzyme activity was seen. In contrast, the mutation S20A resulted in diminished acyltransferase activity regardless of the method of renaturation. Unlike His23, their respective mutations did not lead to total loss of activity; nevertheless, two of the three conserved serine residues appear to be required for full activity. PMSF and AEBSF failed to impair protein activity, but these reagents are known to act only on particularly reactive serine residues. The conserved serine residues in HlyC do not appear to belong to this highly reactive class. Actually, the malonyl/acetyl-

transferase activity of mammalian fatty acid synthase, a member of the proposed GXSXG family of enzymes whose chemical mechanism entails an acyl-serine intermediate, is not inhibited by PMSF, but replacement of the active-site serine with alanine completely eliminates catalytic activity (*28*). With the same enzyme, DEPC sensitivity and site-directed mutagenesis show that a particular histidine residue plays an essential acid−base role in the catalytic mechanism, and mutation of this histidine residue to alanine results in markedly diminished enzyme activity.

Chemical modification and site-directed mutagenesis of HlyC gave analogous results. The HlyCs used in the investigations, however, bore slightly different affinity tags. His_6−S-tag−HlyC was used in site-directed mutagenesis studies and S-tag−HlyC was used in chemical modification experiments. HlyC fusions with both affinity tags exhibited equal expressions and specific activities. Enzymatic removal of the S-tag from S-tag−HlyC shows that it has no effect on enzymatic activity (*8*). Systematic studies of the effects of affinity tags on relatively small proteins show that they are of virtually no consequence regarding biophysical properties (*29* and references therein).

Many acyltransferases, esterases, lipases, and proteinases have a histidine residue intimately involved in catalysis, virtually always in an acid−base role in conjunction with a serine or cysteine 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 (*28*). Although histidine residues are important in many enzyme-catalyzed acyl group transfer reactions, they have not been shown to be a site of acylation in acyl-enzyme intermediates. A histidine residue has been shown, however, to act as a nucleophile forming an acyl-antibody intermediate in a reaction catalyzed by an antibody (*30*). 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. It is unlikely that serine or cysteine residues that are not conserved would have a critical catalytic function. Perhaps conserved residues, separate from histidine, other than those investigated participate in catalysis in this unusual acyltransferase.

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