HlyC, the Internal Protein Acyltransferase That Activates Hemolysin Toxin: The Role of Conserved Tyrosine and Arginine Residues in Enzymatic Activity As Probed by Chemical Modification and Site-Directed Mutagenesis†

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ABSTRACT: Internal fatty acylation of proteins is a recognized means of modifying biological behavior. *Escherichia coli* hemolysin A (HlyA), a toxic protein, is transcribed as a nontoxic protein and made toxic by internal acylation of two lysine residue ϵ-amino groups; HlyC catalyzes the acyl transfer from acyl−acyl carrier protein (ACP), the obligate acyl donor. Conserved residues among the respective homologous C proteins that activate 13 different RTX (repeats in toxin) toxins of which HlyA is the prototype likely include some residues that are important in catalysis. Possible roles of two conserved tyrosines and two conserved arginines were investigated by noting the effects of chemical modifiers and site-directed mutagenesis. TNM modification of HlyC at pH 8.0 led to extensive inhibition that was prevented by the presence of the substrate myristoyl-ACP but not by the product, ACPSH. NAI had no effect. Y70G and Y150G greatly diminished enzyme activity, whereas mutations Y70F and Y150F exhibited wild-type activity. Modification of arginine residues with PG markedly lowered acyltransferase activity with moderate protection by both myristoyl-ACP and ACPSH. Under optimum conditions, four separate mutations of the two conserved arginine residues (R24A, R24K, R87A, and R87K) had little effect on acyltransferase activity.

Internal fatty acylation of proteins is a means of modifying the biological behavior of a protein. For example, lipid modification of proteins influences protein−protein interaction, thus enabling or obviating signal transduction. 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 post-translational protein modification by internal fatty acylation through amide linkage have been reported (*3*, and references therein, *4*−*6*), the enzymes have been neither isolated nor characterized.

Notably, the toxicity of hemolysin (HlyA),[1] a protein toxin secreted by pathogenic *Escherichia coli*, results from the post-translational acylation of two internal lysine ϵ-amino groups. HlyA binds to and lyses mammalian target cell membranes and, at lower concentrations, perturbs cell signal transduction and release of inflammatory mediators (*7*, *8*). It is one of a family of homologous, membrane-active toxins, RTX toxins (repeats in toxin), produced by different Gram-negative bacteria (*9*−*11*). 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 (*12*−*15*). Acyl−acyl carrier protein (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 in forming toxic HlyA and characterized the acyltransferase, HlyC, and the reaction it catalyzes (*15*). A reactive acyl−HlyC intermediate is formed (*15*), and the kinetic mechanism of the HlyC-catalyzed internal acylation of proHlyA was shown to be a uni uni iso uni uni ping pong mechanism.[2] The chemical mechanism of catalysis has not been identified for any protein internal acyltransferase.

Comparison of deduced amino acid sequences of 13 RTX C proteins (Figure 1) reveals extensive homology (*16*). Of the 170 residues in HlyC, 36 are identical among the 13

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[1] Abbreviations: proHlyA, hemolysin A protoxin; HlyA, hemolysin A toxin; RTX, repeats in toxin; HlyC, acyl-ACP-proHlyA 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; acyl-ACP, acyl carrier protein with a long chain fatty acyl covalently attached to the prosthetic group thiol; Hepes, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; PCR, polymerase chain reaction; NAI, *N*-acetylimidazole; TNM, tetranitromethane; PG, phenylglyoxal.

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

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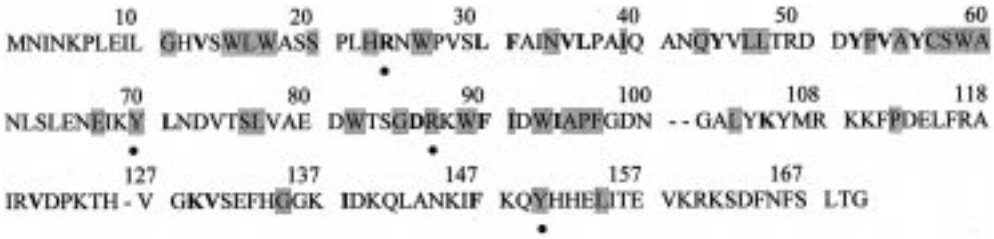

FIGURE 1: Amino acids conserved among 13 RTX toxin C proteins. Residue numbers refer to *E. coli* HlyC encoded by pHly 152. Nonconserved amino acids are shown in uppercase letters; residues conserved with regard to type are shown in bold uppercase letters. Amino acids that are identical in all 13 RTX C proteins are shaded. Breaks introduced to maximize homology are denoted by −. Residues mutated in this manuscript are denoted by ●. Amino acid sequences were deduced from the following DNA sources: HLYC_ECOLI, *E. coli* HlyC, pHly152-encoded, accession number p06736; HLC1_ECOLI, *E. coli* strain J96 HlyC, chromosome-encoded, accession number p09984; HLC2_ECOLI, *E. coli* strain 200l HlyC, chromosome-encoded, accession number p09985; HLYCEH, *E. coli* 0157:H7 strain EDL 933 HlyC, plasmid-encoded, translation of nucleotide sequence accession number x80891; RT1C_ACTPL, *Actinobacillus pleuropneumoniae* Apx-IC, accession number p55132; RT2C_ACTPL, *A. pleuropneumoniae* Apx-IIC, accession number p15376; RT3C_ACTPL, *A. pleuropneumoniae*, Apx-IIIC, accession number q04474; HLYC_ACTAC, *Actinobacillus actinomycetemcomitans* LktC, accession number p16461; HLC1_PASHA, *Pasteunella haemolytica* serotype A1 LktC, accession number p16533; HLC3_PASHA, *P. haemolytica* sertoype T3 LktC, accession number p55120; HLCB_PASHA, *P. haemolytica* serotype A11 LktC, accession number p55121; HLYC_PASSP, *P. haemolytica*-like sp. LktC, accession number p55124; CYAC, *Bordetella pertussis* CyaC, translation of nucleotide sequence accession number m57286. The sequence alignment was done with the Hibio Prosis protein analysis software by Hitachi.

homologous RTX C proteins. Among the conserved residues were several residues that are catalytically important in other types of acyl transfer reactions. A conserved residue is likely the site of acylation during the transient formation of the acyl−enzyme intermediate. We have investigated, via chemical modifications and site-directed mutagenesis, the importance of conserved cysteine, serine, and histidine residues in HlyC catalysis, and a single conserved histidine residue was essential for activity (*17*). In addition to these conserved residues, two tyrosines and two arginines, residues with chemical groups which have been implicated for special roles in many enzymatic reactions (*18*−*21*), are identical, Tyr70, Tyr150, and Arg87, or conserved with respect to type, Arg24 (Figure 1). Chemical modification and site-directed mutagenesis of the conserved tyrosine and arginine residues were used to assess their significance in HlyC catalysis.

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Myristate was from New England Nuclear. Sigma was the source of tetranitromethane (TNM), phenylglyoxal (PG), 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. Urea-containing buffers were always freshly prepared. *N*-Acetylimidazole (NAI) was prepared from acetic anhydride and imidazole as described by Staab and Rohr (*22*). The product was dissolved in warm acetone and precipitated with petroleum ether, and the crystals were dried and stored desiccated at −20 °C.

Bacterial Strains, Media, and DNA Manipulations. E. coli strains were BL21(DE3)pLysS and NovaBlue from Novagen and XL-2 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 an A_{600nm} of 0.6, and harvested after 3 h. Oligonucleotides used for subcloning of the native *hlyC* gene or site-directed mutagenesis of *hlyC* were from Integrated DNA Technologies.

Plasmids encoding two different N-terminal tags in forming two HlyC fusion proteins were used. HlyC was expressed as S-tag-HlyC using pTXC1 as previously described (*15*). HlyC was also expressed as a His_6−S-tag fusion protein from pTXC2 which was constructed as described by Trent and colleagues (*17*). The expression of the HlyC fusion protein was similar from each plasmid, and the specific activities, kinetic parameters, and characteristics of the two fusion proteins were indistinguishable.

Site-Directed Mutagenesis. Site-directed mutations in *hlyC* were generated by the round circle polymerase chain reaction (PCR) method described in the QuikChange Site-Directed Mutagenesis Kit protocol (Stratagene) using the plasmid pTXC2 as the reaction template. The rationale involved whole-plasmid PCR amplification using the mutagenic oligonucleotides shown in Table 1, one upper and lower primer for each mutation. The 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. Mutation of DNA was confirmed by DNA sequence analysis (*23*) at the Molecular Genetics Facility at the University of Georgia (Athens, GA). Plasmids containing mutant HlyC inserts were designated pTXC2 along with a description of the mutation, and the vectors containing mutant HlyCs were transformed into BL21(DE3)pLysS for expression.

Proteins. Protein manipulations were carried out at 4 °C unless noted otherwise. ProHlyA, ACPSH, and radiolabeled myristoyl-ACP were obtained as described by Trent and colleagues (*15*). Myristoyl-ACP was purified and evaluated as described previously (*24*) and stored in aliquots at −80 °C. The fusion protein N-terminal S-tag−HlyC was expressed and purified as previously described (*15*); it was used for the chemical modification studies presented herein. Another HlyC fusion protein, an N-terminal His_6−S-tag−HlyC whose construction and expression are cited above, was employed for site-directed mutagenesis. The His_6−S-tag−HlyC subclone expressed and exhibited kinetics similar to those of the S-tag−HlyC fusion protein that has been extensively characterized (*15*).

Inclusion bodies from either pTXC1 or pTXC2 cells were isolated from the cell lysate pellet (200 mL culture) collected by centrifugation at 20000*g* for 20 min. Inclusion bodies were

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

primer	sequence (5′ → 3′)[a]
R24A upper	GGGCCAGTTCTCCACTACACGCTAACTGGCCAGTATCTTTG
R24A lower	CAAAGATACTGGCCAGTTAGCGTGTAGTGGAGAACTGGCCC
R24K upper	GGGCCAGTTCTCCACTACACAAAAACTGGCCAGTATCTTTG
R24K lower	CAAAGATACTGGCCAGTTTTTGTGTAGTGGAGAACTGGCCC
Y70G upper	GTTTAGAAAATGAAATTAAAGGTCTTAATGATGTTACCTCATTAG
Y70G lower	CTAATGAGGTAACATCATTAAGACCTTTAATTTCATTTTCTAAAC
Y70F upper	GTTTAGAAAATGAAATTAAATTCCTTAATGATGTTACCTCATTAG
Y70F lower	CTAATGAGGTAACATCATTAAGGAATTTAATTTCATTTTCTAAAC
R87A upper	CAGAAGACTGGACTTCAGGTGATGCTAAATGGTTCATTGACTGG
R87A lower	CCAGTCAATGAACCATTTAGCATCACCTGAAGTCCAGTCTTCTG
R87K upper	GCAGAAGACTGGACTTCAGGTGATAAAAAATGGTTCATTGACTGG
R87K lower	CCAGTCAATGAACCATTTTTTATCACCTGAAGTCCAGTCTTCTGC
Y150G upper	GCGAATAAAATTTTTAAACAAGGTCACCACGAGTTAATAACTG
Y150G lower	CAGTTATTAACTCGTGGTGACCTTGTTTAAAAATTTTATTCGC
Y150F upper	GCGAATAAAATTTTTAAACAATTCCACCACGAGTTAATAACTGAAG
Y150F lower	CTTCAGTTATTAACTCGTGGTGGAATTGTTTAAAAATTTTATTCGC

[a] Underlined codons encode amino acid changes.

washed three times with 25 mL portions of 25 mM Hepes (pH 8), 5 mM EDTA, and 2 mM DTT. HlyC was solubilized by suspending the inclusion bodies in 15 mL of 25 mM Hepes (pH 8), 5 mM EDTA, 2 mM DTT, and 6 M urea for 1.5 h. The fusion protein solution was clarified by centrifugation at 30000*g* for 30 min.

Routinely, protein from inclusion bodies obtained from 200 mL cultures of pTXC1 or pTXC2 cells was slowly refolded by stepwise removal of urea by dialysis (M_r cutoff of 8). The protein solution in 6 M urea buffer was dialyzed against 1.0 L of 25 mM Hepes (pH 8), 5 mM EDTA, and 2 mM DTT containing 3 M and then 1 M urea, each for 3 h, and finally against 25 mM Hepes (pH 8), 5 mM EDTA, and 2 mM DTT for 16 h. The solution was clarified by centrifugation at 30000*g* for 30 min. The fusion protein was precipitated overnight at 65% $(NH_4)_2SO_4$ saturation, collected by centrifugation at 30000*g* for 30 min, and dissolved in 25 mM Hepes (pH 8), 5 mM EDTA, and 2 mM DTT. It was stored in aliquots at −20 °C. The protein yield was ∼2 mg.

Alternatively, urea extracts of inclusion bodies containing wild-type or mutant His_6−S-tag−HlyC were subjected to quick refolding as described subsequently. This was done so possible structural roles of mutant residues could be detected. Mutant and wild-type cultures of 5 mL were grown simultaneously, induced, harvested, and stored at −20 °C. At the time of use, cells were thawed and disrupted as previously described (*15*), and with subsequent manipulations at 4 °C, 30 mL of 25 mM Hepes (pH 8.0), 5 mM EDTA, and 1 mM DTT was added to each batch of cells to wash the inclusion bodies. The supernatant solutions were removed by centrifugation for 15 min at 30000*g*, and pellets were suspended in the above buffer that contained 6 M urea. After 1 h with occasional agitation, the supernatant solutions were collected by centrifugation for 20 min at 30000*g* and analyzed for protein. The fusion protein, 10 μg from each sample, extracted from inclusion bodies in 6 M urea buffer was brought to 100 μL with 25 mM Hepes (pH 8) and kept on ice for 25 min. At that time, substrates were added, and acyltransferase activity was assayed.

Assays. Protein was assessed as described by Bradford (*25*). The purity of each protein that was used was assessed by densitometry following SDS−PAGE (*26*). HlyC fusion proteins were ∼85% pure, and proHlyA was ∼90% pure. The HlyC-catalyzed transfer of radiolabeled myristate from myristoyl-ACP to proHlyA to form radiolabeled HlyA was assessed as previously described (*15*).

Chemical Modification of HlyC. Modification reactions were initiated by the addition of freshly prepared chemical reagents in a particular solvent to N-terminal S-tag−HlyC isolated from inclusion bodies as described above using the procedure of Trent and colleagues (*15*). For each modification reaction, a control reaction with solvent only was processed in a manner identical to that of the experimental reaction. Following chemical modification, reagent was either removed by $NH_4(SO_4)_2$-induced precipitation of the enzyme or quenched as described below.

Modification of S-tag−HlyC by TNM was carried out at 25 °C by adding an ethanol solution of the reagent (10 mM) to a solution of enzyme, 95 μg in 100 μL of 0.05 M Tris (pH 8.0) or 0.1 M KPO_4 (pH 6.0). Reaction concentrations of TNM ranged from 0.165 to 1.65 mM. Reactions were quenched after 30 min with a 20-fold molar excess of L-tyrosine in 0.05 M Tris (pH 8.0) buffer and assayed for acyltransferase activity. To measure the extent of modification with time, a larger reaction mixture was prepared, and at intervals, 10 μg aliquots of enzyme solution were removed, quenched, and assayed for enzyme activity. Tyrosine did not affect the assay of acyltransferase activity.

Modification of S-tag−HlyC by NAI entailed adding NAI to final concentrations of a 60−600-fold molar excess relative to HlyC from a freshly prepared 100 mM stock in acetonitrile to 10 μg of S-tag−HlyC in 50 μL of 50 mM Hepes (pH 7.5). After 30 min at 25 °C, NAI was removed by 70% saturation $(NH_4)_2SO_4$ precipitation of S-tag−HlyC. S-tag−HlyC was dissolved in 25 mM Hepes (pH 7.0), and acyltransferase activity was measured at pH 7.0 to avoid reversal of any modification.

PG, 200 mM in 25 mM Hepes (pH 8.0) and 50% ethanol, was added to final concentrations ranging from 0.1 to 5 mM to 10 μg of S-tag−HlyC in 50 μL of 25 mM Hepes (pH 8.0). After 2 h at 25 °C, reactions were quenched with a 20-fold molar excess of L-arginine in 25 mM Hepes (pH 8.0) and assayed for acyltransferase activity. Arginine did not affect the assay of S-tag−HlyC activity.

Spectral Measurements. Reaction of S-tag−HlyC with TNM or NAI was monitored spectrally using a Hewlett-Packard 8452A diode array spectrophotometer scanning at 2 nm increments at wavelengths ranging from 200 to 500

nm or a Gilford 240 spectrophotometer. One milliliter of HlyC (22.3 μM) in 0.05 M Tris (pH 8.0) was treated in a 1 cm cuvette with 1 mM TNM; spectra were taken prior to TNM addition and after intervals of treatment with TNM at 25 °C. Nitroformate anion, generated during TNM reaction with proteins, was removed using a Centricon (Amicon) 10 000 molecular weight cutoff. The increase in absorbance at 428 nm during TNM treatment was used to calculate the moles of 3-nitrotyrosine formed using an ϵ_{428nm} of 4200 (*27*). Using the HlyC concentration given above, the number of moles of 3-nitrotyrosines formed per mole of HlyC was calculated.

Two 1 mL aliquots of HlyC (55.8 μM) in 25 mM Hepes (pH 7.5), one treated with a 60-fold excess of NAI in acetonitrile and one with solvent only (2.2 μL of acetonitrile), were mixed together. After 1 h at 25 °C, both solutions were dialyzed again a 250 mL portion of Hepes buffer (pH 7.5), four changes over 20 h, after which spectra of both solutions were measured. The absorbance decrease at 278 nm was used to calculate the amount of *O*-acetyltyrosine formed [$\Delta\epsilon_{278nm} = 1160\ M^{-1}\ cm^{-1}$ (*28*)]. Using the HlyC concentration given, the number of moles of acetylated tyrosines per mole of HlyC was calculated.

Effects of Myristoyl-ACP and ACPSH on HlyC Modification by TNM and PG. To investigate the ability of myristoyl-ACP or ACPSH to protect S-tag−HlyC against modification by TNM or PG, S-tag−HlyC (2 μM) was incubated for 5 min with either unlabeled myristoyl-ACP or ACPSH (2 μM) followed by treatment with either TNM or PG as described above. Following reaction, samples were precipitated at 70% $(NH_4)_2SO_4$ saturation at pH 7.0 and 4 °C, and S-tag−HlyC was collected by centrifugation and dissolved. Neither myristoyl-ACP nor ACPSH precipitates under these conditions; therefore, all ACP was removed prior to assembling the S-tag−HlyC for assay. Acyltransferase activity was then measured as previously described (*15*).

RESULTS

Effects of Tetranitromethane and N-Acetylimidazole. HlyC contains seven tyrosine residues, and two are conserved among RTX C proteins (Figure 1). TNM is an efficient and specific reagent for the nitration of solvent-accessible tyrosine residues (*27*, *29*). It reacts with tyrosine residues at pH 8.0 with high specificity to form 3-nitrotyrosine, an ionizable nitrated tyrosine residue with singular spectral characteristics (*30*). The reaction is pH-dependent with no modification of tyrosine occurring at pH 6.0 where the reagent oxidizes sulfhydryl groups. In the absence of any chemical modification reagent, HlyC activity at pH 6.0 was reduced compared to that observed at pH 8.0 where it is usually assayed (Figure 2A,C), but S-tag−HlyC treated with TNM at pH 6.0 had 82% of the acyltransferase activity of the untreated enzyme at the same pH (Figure 2A,B). In contrast, exposure of HlyC to TNM at pH 8.0 resulted in the diminution of acyltransferase activity to 28% of that of the untreated enzyme (Figure 2C,D). TNM inhibition of S-tag−HlyC at pH 8.0 was dependent upon the concentration of the inhibitor, and inactivation of acyltransferase activity followed first-order kinetics (Figure 3A,B). In the absence of inhibitor, the conditions did not impair enzyme activity as shown by the level plot of control activity in Figure 3B. The pH dependence of TNM inhibition of S-tag−HlyC indicated modification of tyrosine residue(s) by the reagent, and changes in S-tag−HlyC's absorbance spectrum upon treatment with TNM verified the formation of 3-nitrotyrosine (Figure 4). S-tag-HlyC exhibited an absorbance peak at 428 nm following exposure to TNM and subsequent removal of nitroformate ion which absorbs strongly at 350 nm. The acyltransferase activity decline corresponded to the increase in absorbance of S-tag−HlyC at 428 nm, which exhibited the formation of 3-nitrotyrosine residues (Figure 5). Using the pH-sensitive molar absorption coefficient of 4200 for 3-nitrotyrosine residues (*30*), 2.3 mol of tyrosine residue was modified per mole of S-tag−HlyC upon exposure to TNM.

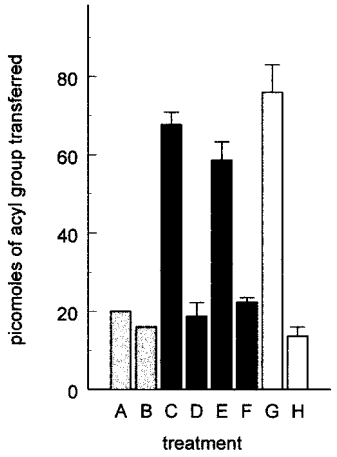

FIGURE 2: Effects of TNM and NAI on HlyC activity under various conditions. The methods of exposing S-tag−HlyC to TNM and/or NAI under various conditions of pH and in the presence and absence of substrate or product and inhibitor removal are given in Experimental Procedures. Acyltransferase activity was assayed as previously described (*15*). Assays were carried out at the following different pHs required to prevent removal of any inhibitor covalently bound to HlyC: pH 6.0, light gray bars; pH 8.0, dark gray bars; and pH 7.0, white bars. Controls were exposed to solvents and handling similar to those of the corresponding experiments. The bars represent the average acyltransferase activities (error bars represent the standard deviation) of HlyC treated with inhibitors under the following conditions: (A) pH 6.0 control, (B) 1.55 mM TNM, (C) pH 8.0 control, (D) 1.55 mM TNM, (E) 1.55 mM TNM in the presence of myristoyl-ACP, (F) 1.55 mM TNM in the presence of ACPSH, (G) 3 mM NAI, and (H) 3 mM NAI followed by 3 mM TNM.

The presence of the substrate myristoyl-ACP during exposure of the enzyme to TNM at pH 8 resulted in almost complete protection of acyltransferase activity (90 ± 8% of the untreated control). This is shown in Figure 2 where E shows the activity seen when myristoyl-ACP was present during TNM treatment compared to D, which shows the activity of S-tag−HlyC exposed to TNM without substrate protection, and C, which shows the activity of S-tag−HlyC exposed to solvent only. The presence of product, ACPSH, during treatment of S-tag−HlyC with TNM afforded little or no protection against the action of the reagent on the enzyme (Figure 2F). Enzyme exposed to TNM in the presence of ACPSH had 33 ± 2% of the activity of untreated enzyme, almost the same percentage as the enzyme exposed to TNM in the absence of substrate or product which was 28 ± 5%.

Treatment with TNM has been shown to cause aggregation of some proteins secondary to the generation of intermolecular cross-linkages between tyrosine residues (*31*). Ex-

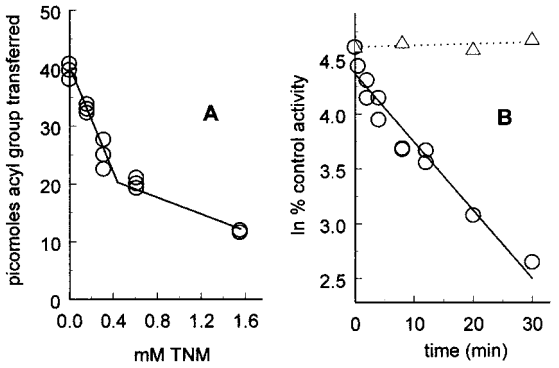

FIGURE 3: TNM effects on acyltransferase activity at pH 8.0. Acyltransferase activity was assayed as previously described (*15*). The data points are the results of individual assays. Graph A shows enzyme activity following treatment with different concentrations of TNM. Preparation of S-tag−HlyC and treatment with different concentrations of TNM were carried out as described in Experimental Procedures. Graph B shows a linear regression plot of the natural log of the percentage of the control activity relative to the time of exposure to 1.55 mM TNM (○). A solution of HlyC, 220 μg in 500 μL of 0.05 M Tris (pH 8.0), was prepared. One hundred microliters was removed for solvent control assays, and two 23 μL aliquots were placed in assay tubes containing 20 μL of 10 mM tyrosine in 0.05 M Tris (pH 8.0) (for reaction quenching). These were 0 time acyltransferase assay controls. The larger aliquot of the enzyme solution was treated with 1 mM TNM at 25 °C, and 23 μL aliquots were removed at the indicated times for quenching and enzyme assay. The 100 μL aliquot was treated with an equal volume of solvent only, and 23 μL aliquots were withdrawn at the indicated times for measurements of activity of enzyme not exposed to TNM (△). Enzyme activity from individual assays is shown as ln % of control activity which was 53.5 ± 4.0 pmol of acyl groups transferred.

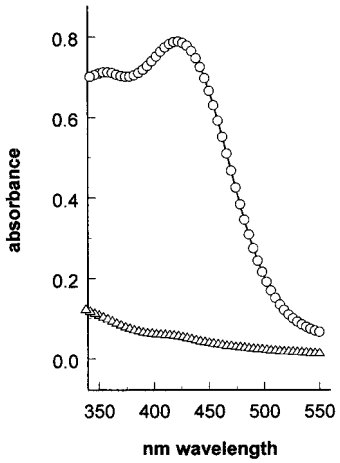

FIGURE 4: Spectra of unmodified and TNM-modified HlyC. Spectra of 22.3 μM HlyC were recorded before and after modification with 1.55 mM TNM. Details are given in Experimental Procedures.

amination of S-tag−HlyC by SDS−PAGE following treatment with TNM did not show the formation of any HlyC aggregates (not shown). This finding ensured that the diminution in HlyC activity observed upon exposure to TNM did not stem from protein aggregation.

Acetylation with NAI is a means of modifying phenolic hydroxyl groups of tyrosine residues under mild conditions (*28*). Exposure of S-tag−HlyC to NAI up to a 600-fold excess had no effect on acyltransferase activity (Figure 2G). Since the reagent acetylates the hydroxyl groups of tyrosyl residues, it causes a decrease in the A_{278nm} of proteins. Although activity was not impaired, the absorbance at 278

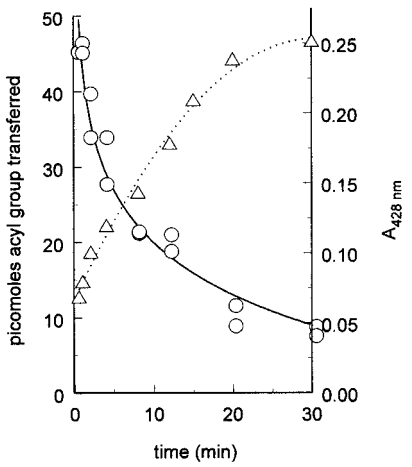

FIGURE 5: Relationship between acyltransferase activity and extent of tyrosine modification upon exposure of HlyC to TNM. To observe the decrease in enzyme activity with time (○), a solution of HlyC was prepared and processed as described in the legend of Figure 3B. Acyltransferase was assayed as previously described (*15*); the data points are the results of individual assays. Details of the spectrophotometric measurements are given in Experimental Procedures; absorbance values are shown as △.

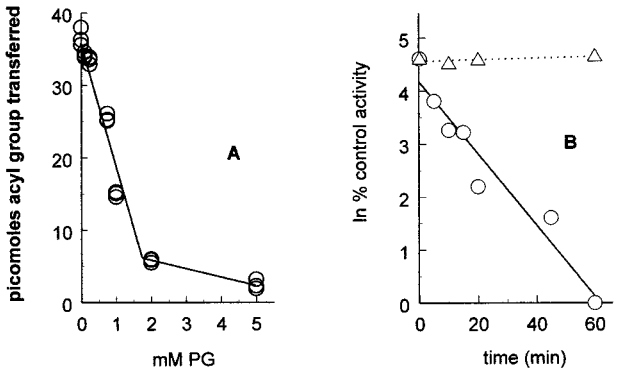

FIGURE 6: PG effects on HlyC activity. Acyltransferase activity was assayed as previously described (*15*). The data points are the results of individual assays. Graph A shows the concentration dependence of the PG effect on HlyC activity. Preparation of S-tag−HlyC and treatment with different concentrations of PG were carried out as described in Experimental Procedures. Graph B shows a linear regression plot of the natural log of the percentage of control activity at increasing exposure time to 5 mM PG (○). The control activity was 34.3 ± 2.9 pmol of acyl groups transferred. A series of control assays were treated identically except that PG was omitted (△).

nm of 1.0 mL of 55.8 μM S-tag−HlyC decreased by 0.071 upon treatment with NAI. Using the molar absorption coefficient for *O*-acetyltyrosine formation $\Delta\epsilon$ of 1160 (*28*), 1.1 mol of tyrosyl residues/mol HlyC reacted with the reagent. S-tag−HlyC that was first reacted with NAI was subsequently exposed to TNM with which it also reacted, as indicated by the extensive inhibition depicted in Figure 2H.

Effects of Phenylgloxal. PG reacts specifically with arginine groups in proteins under mild conditions (*32*). Of HlyC's seven arginine residues, one is conserved and one is conserved regarding residue type (Figure 1). Exposure of S-tag−HlyC to increasing concentrations of PG resulted in linearly increasing inhibition of acyltransferase activity up to 2 mM PG, where activity was reduced to ∼20% of that observed in assays which contained no reagent (Figure 6A). The PG concentration-dependent inhibition of S-tag−HlyC was biphasic, and by 5 mM PG, S-tag−HlyC was inhibited

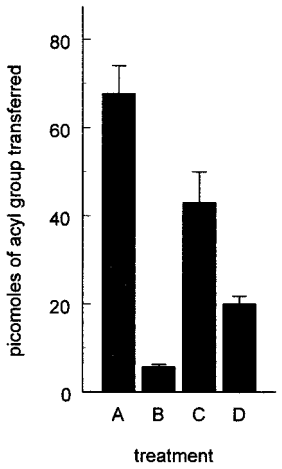

FIGURE 7: Effects of PG on HlyC activity under different conditions. The details of the treatment of S-tag−HlyC with 5 mM PG under various conditions are given in Experimental Procedures. The control was exposed to solvents and handling similar to those of the corresponding experiments. The bars represent the average acyltransferase activities (error bars represent the standard deviation) of HlyC treated with PG under the following conditions: (A) control, (B) 5 mM PG, (C) 5 mM PG in the presence of myristoyl-ACP, and (D) 5 mM PG in the presence of ACPSH.

to 8 ± 1% of the activity measured in the absence of reagent (Figures 6A and 7A,B). Incubating S-tag−HlyC with substrate or product prior to treatment altered the enzyme's susceptibility to PG (Figure 7C,D). In contrast to ACPSH's lack of protection against TNM inhibition of S-tag−HlyC, the presence of the reaction product afforded a slight but credible protection against PG inhibition (Figure 7D compared to Figure 7B). With ACPSH present upon exposure to PG, acyltransferase activity was 29 ± 2% of the control, whereas with no ACPSH protection, the level of PG inhibition was 8 ± 1% of the control. When S-tag−HlyC was incubated with myristoyl-ACP, the degree of protection from PG was greater than that seen with product, 68 ± 13% of the control activity (Figure 7C). This protection was not, however, of the degree of protection that substrate provided against TNM inhibition where 90 ± 8% of the control activity was retained.

Mutation of Conserved Tyrosine and Arginine Residues. The following site-directed mutations of N-terminal His_6−S-tag−HlyC were confirmed by DNA sequence analysis: Y70G, Y70F, Y150G, Y150F, R24A, R24K, R87A, and R87K. The mutant HlyCs was expressed and purified like the wild-type His_6−S-tag−HlyC. Mutants were slowly refolded by dialysis following enzyme extraction from inclusion bodies into buffered 6 M urea as described in Experimental Procedures. For comparison, mutants were also quickly renatured by diluting the enzyme freshly extracted in buffered 6 M urea from inclusion bodies ~25-fold to decrease the urea concentration quickly to <0.3 M, and after 25 min at 4 °C, acyltransferase activity was measured. The acyltransferase activities stemming from the each of the two methods used to renature each mutant are compared in Table 2 with similar refolding of wild-type HlyC.

Substitution of Tyr70 with glycine resulted in the loss of virtually all enzyme activity (Table 2). To distinguish between the contribution of a large, hydrophobic residue and potential specific participation of the phenolic hydroxyl group, the effects of substituting phenylalanine for Tyr70 on acyltransferase activity were examined. Consequent to such substitution, Y70F enzyme activity was restored to that of the wild-type enzyme upon slow refolding of HlyC (Table 2). If, however, the Y70F mutant was quickly refolded, it was a less effective enzyme compared with its activity upon slow refolding or with the activity of quickly renatured wild-type HlyC.

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

	% of native acyltransferase activity	
mutant	quick dilution	slowly refolded
Y70G	10 ± 1.1	12 ± 0.1
Y70F	75 ± 5.2	106 ± 1.4
Y150G	21 ± 1.2	44 ± 0.7
Y150F	92 ± 1.7	86 ± 6.4
R24A	67 ± 1.8	64 ± 0.6
R24K	100 ± 5.0	not done
R87A	18 ± 0.6	82 ± 5.5
R87K	53.8 ± 5.7	99 ± 9.2

[a] Acyltransferase activity was measured as described in Experimental Procedures. Native slowly refolded HlyC activity was 14.4 nmol of acyl groups transferred/mg of enzyme. Native quick dilution HlyC activity was 6.6 nmol of acyl groups transferred/mg of enzyme.

Mutation of the other tyrosine residue conserved among all RTX C proteins, Tyr150, to glycine resulted in HlyC with impaired acyltransferase activity (Table 2). Y150G had, upon slow refolding, about half the activity of the wild-type His_6−S-tag−HlyC. Quick renaturation of enzyme following urea extraction from inclusion bodies resulted in even less activity. Restitution of residue type, but not functional group, with the mutation Y150F resulted in restoration of acyltransferase activity to wild-type levels regardless of the refolding method. Treatment of either slowly refolded functional mutant of the two conserved tyrosines, Y70F or Y150F, with TNM resulted in inhibition of enzyme activity like that reported above (Figure 1). In the same vein, myristoyl-ACP protected both Y70F and Y150F from TNM inhibition to the same extent as that observed for protection of the wild type. Like the wild type, neither mutant was protected by ACPSH from TNM inhibition. Thus, the conserved tyrosine residue mutants, Y70F and Y150F, as evidenced by acyltransferase activity, are each equally affected by TNM, equally protected from TNM by substrate, and equally unprotected by product. TNM modification of Y70F and Y150F did not distinguish separate biological roles for the two conserved tyrosine residues.

Substitution of the positively charged Arg24 with alanine resulted in an enzyme with almost half the activity of wild-type His_6−S-tag−HlyC (Table 2), regardless of the refolding method. If the positive charge was preserved at the position, R24K, activity was unimpaired. Mutation R87A, however, did not alter the activity of slowly refolded enzyme compared to the wild type, whereas it was greatly impaired in activity when subjected to quick renaturation. This observation implicated the importance of positive charge on Arg87 in guiding the refolding of denatured His_6−S-tag−HlyC. A positive charge was provided at residue 87 by substituting a lysine for Arg87. The increased activity of the quickly diluted R87K mutant to half of the wild-type enzyme activity compared to R87A (one-fifth as active as the wild type) indicated that a positively charged residue at position 87 of the primary sequence contributes to the efficacy of His_6−

S-tag−HlyC refolding (Table 2). Each of the slowly refolded arginine to alanine mutants listed in Table 2 was, like wild-type enzyme, inhibited by PG; the effects of myristoyl-ACP or ACPSH on these inhibitions were not examined.

DISCUSSION

Chemical modification experiments and results of site-directed mutagenesis of selected conserved residues have shown that the two arginines and two tyrosines conserved among RTX C proteins are not as such essential for acyltransferase activity. Their modifications do, however, result in impaired acyltransferase activity compared to wild-type enzyme. A residue may be crucial to enzymatic function 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 suggest that reactive serine residues, cysteine residues, or amine groups are not required for acyltransferase activity (*17*). The lack of response to TNM at pH 6.0 verified that the single conserved cysteine has no essential role in catalysis as reported previously (*17*); TNM at pH 6.0 oxidizes sulfhydryl groups (*33*). In contrast, the behavior of the enzyme upon treatment with DEPC at pH 6.0 shows that the integrity of histidine residue(s) is essential for activity (*17*). HlyC forms a reactive acyl−enzyme intermediate (*15*); the nature of the residue acylated is not known.

Modification of tyrosyl groups with TNM to form 3-nitrotyrosine residues resulted in the loss of more than 80% of S-tag−HlyC acyltransferase activity, suggesting the functional importance of one or more tyrosine residues. HlyC contains seven tyrosine residues, two of which are conserved among RTX C proteins. Chemical modification with NAI, which acetylated the phenolic hydroxyl group of tyrosine rather than modifying the aromatic ring like TNM, did not impair enzyme activity. Enzyme activity was greatly impaired when the fully active NAI-modified enzyme was substituted by treatment with TNM. Although it has not been shown, tyrosine residues accessible to TNM would likely be equally accessible to NAI. The results of chemical modifications of tyrosine residues indicated that the integrity of the hydroxyl group of at least one residue was not critical to acyltransferase activity. This finding agrees with an earlier observation where treatment with diethyl pyrocarbonate under conditions where tyrosine hydroxyl group(s) could be modified does not affect acyltransferase activity either (*17*).

When HlyC was treated with TNM in the presence of substrate myristoyl-ACP, tyrosine residue(s) responsible for the loss of activity were protected from access by the reagent, and acyltransferase activity was largely preserved. If, instead, the reaction product ACPSH was present when HlyC was treated with TNM, 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 predicts an isomerization of the enzyme, and consequently, acyl-ACP and ACPSH bind to different forms of HlyC. Protection by substrate to this extent suggests involvement of the modified tyrosine residue(s) in or proximal to the catalytic site.

PG is highly selective for arginine residues (*34*), and treatment of S-tag−HlyC with PG virtually abolished acyltransferase activity. HlyC contains seven arginine residues. Myristoyl-ACP, however, provided acyltransferase activity less protection from PG inhibition than it did from TNM inhibition. Also, there was less difference between the amount of protection from PG inhibition conferred by substrate presence compared to that conferred by product presence. The effects of PG on enzyme activity seemed generally less incisive than the actions of other inhibitors that affected enzyme activity, TNM and diethyl pyrocarbonate (*17*).

Individual mutagenesis of evolutionarily conserved residues of HlyC augmented the results of chemical modification studies. Of the conserved tyrosine and arginine residues that were mutated, only one mutation led to a complete loss of activity, Y70G. An alternative site-directed replacement, Y70F, revealed that a tyrosyl residue per se at position 70 was not essential for acyltransferase activity, providing a large, hydrophobic residue was present; Y70F when gradually refolded fully restored wild-type activity. The slightly impaired activity of quickly refolded Y70F implied a role for the residue that was optimal when a hydroxyl group was present on the aromatic ring. Specific site mutations of Tyr150, Y150G and Y150F, led to similar although less pronounced findings regarding enzyme activity; the glycine mutant was active, but the degree of activity was dependent upon refolding speed. Substitution with a large, hydrophobic residue restored wild-type activity regardless of the refolding method. Acyltransferase activity was not as sensitive to mutation of Tyr150 as it was to mutation of the other conserved tyrosine, Tyr70.

Substitution of either of the two conserved tyrosine residues with phenylalanine did not protect HlyC from being inhibited by TNM to the same extent as the wild-type enzyme. Which of HlyC's seven tyrosine residues reacted with TNM is not known. Substrate and product protection experiments for each of the two tyrosine mutants of His_6−S-tag−HlyC, Y70F and Y150F, gave results for each mutant similar to those seen with the wild-type enzyme. Myristoyl-ACP largely protected TNM from inhibition, while ACPSH did not. This observation suggests that the TNM-modified tyrosines that were important for HlyC's activity are situated in or near the catalytic site, but it does not distinguish between conserved and unconserved tyrosine residues. Perhaps the bulky, hydrophobic residue participates in positioning the substrate; the acyl chain of myristoyl-ACP would likely require a hydrophobic milieu for alignment within the catalytic site prior to transfer to form an acyl−HlyC intermediate. Fatty acid and acyl-CoA binding proteins that have been examined contain aromatic residues in the fatty acid binding cavities (*35*, *36*). Aromatic residues are involved in the positioning of the acyl group in the tertiary structure of acyl-ACP (*37*−*39*). Protection of specific tyrosine residues of both urokinase-type plasminogen activator and its receptor while they were complexed from TNM modification elegantly demonstrated the validity of ligand protection as an indicator of intermolecular interfacing and, notably, the involvement of specific tyrosine residues in ligand binding (*20*).

Mutation of the two conserved arginines did not inactivate HlyC, although R24A did reduce the activity while slowly refolded mutants of Arg87 exhibited wild-type enzyme activity. PG inhibition/substrate product protection experi-

ments and site-directed mutagenesis indicated that the two conserved arginines did not have a critical role, if indeed any, in the active site vicinity of HlyC. R87K was a more effective enzyme upon quick refolding than was R87A. The presence of a positive charge at residue 87 appeared to assist refolding of denatured HlyC. Arg87 likely has a structural role in the enzyme that has been conserved among RTX C proteins rather than a significant part in catalysis. Arg24 is preserved for residue charge rather than identity among RTX C proteins, and R24K, as anticipated, behaved like the wild-type enzyme.

Although optimum wild-type catalysis ensues when they are present and not modified, neither of the two tyrosines nor the two arginines conserved among RTX C proteins are essential as such for catalysis. An active acyl−enzyme intermediate is shown to be part of the catalytic mechanism (*15*). Although it was a feasible candidate as a site for enzyme acylation, the hydroxyl group of a conserved tyrosine residue(s) was not crucial for that essential step in catalysis without which reaction could not proceed, the formation of acyl-HlyC (*40*). Previous studies have ruled out such a function for the single cysteine, and three serines conserved among RTX C proteins; only the single conserved histidine, His23, is essential for activity among the residues that were examined (*17*). Perhaps conserved residues, separate from histidine and other than those investigated, participate in catalysis in this unusual acyltransferase; it seems unlikely, however, that nonconserved residues among the 13 RTX C proteins would possess such a critical catalytic function.

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