α-Hemolysin (HlyA) is a secreted protein virulence factor observed in certain uropathogenic strains of Escherichia coli. The active, mature form of HlyA is produced by posttranslational modification of the protein that is mediated by acyl carrier protein and an acyltransferase, HlyC. We have now shown using mass spectrometry that these modifications, when observed in protein isolated in vivo, consist of acylation at the ε-amino groups of two internal lysine residues, at positions 564 and 690, with saturated 14- (68%), 15- (26%), and 17- (6%) carbon amide-linked side chains. Thus, HlyA activated in vivo consists of a heterogeneous family of up to nine different covalent structures, and the sub-structure specificity of the HlyC acyltransferase appears to differ from that of the closely related CyaC acyltransferase expressed by Bordetella pertussis.

α-Hemolysin (HlyA,1 110 kDa) is a widely studied protein toxin secreted by uropathogenic strains of Escherichia coli. It is the most studied toxin of the RTX (repeat in toxin) class (1) and is among the most studied toxin of the RTX (repeat in toxin) class (1) and is an important in Gram-negative pathogenesis (2).

Other RTX toxins that have been characterized include adenylate cyclase toxin (ACT, the CyaA protein from Bordetella pertussis) and leukotoxin (LktA) from Pasteurella hemolytica. This class of proteins is defined by a common feature, a repeat region rich in aspartic acid and glycine. They are secreted by pathogens during infection and are known to attack host immune system cells, such as macrophages and lymphocytes. At high concentrations they are lytic for their respective populations of target cells. At sublytic concentrations HlyA is known to disrupt host cell signal transduction and cytokine production (1, 2).

Intracellular activation process is shared by the members of the RTX toxin family (3). In HlyA, for example, the activation process is dependent on the product of an accessory gene, hlyC, and is accomplished by acyl-acyl carrier protein (acyl-ACP) dependent fatty acylation (4, 5). Studies of in vivo activated HlyA protein have revealed that two internal residues,2 Lys-564 and Lys-690, are acylated (6). Analysis of the in vivo activated HlyA protein using two-dimensional gels has confirmed the sites of activation (7) but not the chemical structures of the modifications. The structural details of the fatty acylation of CyaA from B. pertussis have been studied. Active CyaA isolated from B. pertussis was observed to be palmitoylated at only one internal lysine residue, Lys-983, which corresponds to Lys-690 of HlyA (8) according to RTX toxin sequence alignments. Similarly, Lys-860 of CyaA corresponds to Lys-564 of HlyA. However, recombinant CyaA, isolated from E. coli containing cyaA and cyaC genes, is acylated at two sites. Lys-983 is about 87% palmitoylated and the remainder myristoylated, and Lys-860 is approximately two-thirds palmitoylated (9). More recently, it has become clear that acylations observed in CyaA show a high degree of variability, especially at Lys-860. We have now observed Lys-860 acylation, by an as yet uncharacterized side chain, in recent preparations of ACT expressed by B. pertussis (BP338),2 the same strain we first analyzed in 1994 (8) and again in 1995 (9). Similarly, we have observed Lys-860 palmitoylation in a recombinant form of CyaA-expressing E. coli.
expressed in B. pertussis strain 18323/pHSIP9.4. However, CyaA appears to require fatty acylation only at Lys-983 for cell-invasive activity. In contrast to the somewhat more complex set of observations for CyaA, all preparations of native, active HlyA reported to date have probably been fully acylated at both lysines (2). Alterations in fatty acid composition are believed to result in functional changes, and the non-acylated protoxin is inactive in the case of both CyaA (9, 10) and HlyA (6). Interestingly, acylation is not a requirement for secretion of either CyaA or HlyA but is required at both sites in HlyA for full activity as a cytolsin or hemolysin (11). RTX toxin maturation and activation studies up to 1998 (2) have been reviewed in the context of other types of lipid protein modifications.

In order to determine the structural details of fatty acylation present in vivo activated HlyA, we analyzed the proteolytic fragments of HlyA using the techniques of peptide mapping, off-line HPLC, MALDI-TOF mass spectrometry, and electrospray ionization (ESI) mass spectrometry coupled with micro-capillary HPLC, capillary gas chromatography coupled with electron impact mass spectrometry (GC/MS), and peptide synthesis. Two different sources of HlyA, one chromosomal (J96) and the other extrachromosomal (pHly152), and several different preparations from one source (J96) were used in order to control for potential variability between laboratories and individual preparations.

**Experimental Procedures**

**E. coli Strains Used for Toxin Production—**Two forms of activated α-hemolysin were used, HlyA<sub>amo</sub> and HlyA<sub>pHly152</sub>. ProHlyA<sub>amo</sub> (the inactive proteolysin control) and HlyA<sub>pHly152</sub> were prepared by either ammonium sulfate or polyethylene glycol precipitation of LB broth supernatants of E. coli strains WAM783 (12) and WAM1824 (13). Ammonium sulfate was preferred due to the tendency of polyethylene glycol residue to interfere with analysis by mass spectrometry. The hlyA gene in HlyA<sub>amo</sub> is based on the hemolysin recombinant plasmid pSF4000 encoding the hly operon from the pathogenicity island (PAI IV) at 64 min in the chromosome of E. coli uropathogenic strain J96 (14, 15). LB broth cultures (2 liters) were grown at 37 °C with moderate aeration to an absorbance of 1.0 at 600 nm. The acetonitrile was necessary to minimize losses of Hly AJ96 is based on the hemolysin recombinant plasmid pSF4000 encoding the hly operon from the pathogenicity island (PAI IV) at 64 min in the chromosome of E. coli uropathogenic strain J96 (14, 15). LB broth cultures (2 liters) were grown at 37 °C with moderate aeration to an absorbance of 1.0 at 600 nm. The acetonitrile was necessary to minimize losses of Hly AJ96.

**Purification of Toxins—**We purified approximately 500 pmol of HlyA<sub>pHly152</sub> for this study.

**Fatty Acid Analysis—**Both peptide fragments and intact protein were analyzed for fatty acid content measured as fatty acid methyl esters (FAMES) using the direct transmethylation method (25). In addition to C12:0 as a positive control and internal standard, additional positive control and blank experiments were performed concurrently to ensure the validity of the data. GC/MS conditions were as follows: 30 m × 0.25-mm inner diameter BPX5 column (SGE, Austin, TX) with 0.25-μm film thickness, 100–250 °C 15-min temperature gradient on a Hewlett-Packard (Palo Alto, CA) 5890 GC and 7673 autosampler, interfaced with a Trio 2000 quadrupole MS (Micromass, Manchester, UK) 70-eV electron impact ionization. For quantitation, the GC/MS total ion currents for each fatty acid were measured relative to the C12:0 internal standard and corrected for any background level contamination.

**RESULTS AND DISCUSSION**

We had tried unsuccessfully to digest HlyA with trypsin, possibly due to incomplete denaturation of HlyA in 1 M urea. Lys-C, which can tolerate a higher urea concentration (4 M) was a better choice. This was consistent with the difficulties ob-

---

4 Havlicek, V., Higgins, L., Chen, W., Halada, P., Sebo, P., Sakamoto, H., and Hackett, M. (2001) J. Mass Spectrom., in press.
5 Basar, T., Havlicek, V., Bezouskova, S., Hackett, M., and Sebo, P. (2001) J. Biol. Chem., in press.

---

**Microbore HPLC and Screening by MALDI-TOF Mass Spectrometry—**Results from the initial screening indicated which HPLC fractions contained putative modification sites. Those fractions were analyzed by microbore HPLC coupled to a TSQ 7000 electrospray tandem quadrupole mass spectrometer (Finnigan, San Jose, CA). One μl of each Lys-C fraction was eluted from a 50-μm inner diameter × 12-cm capillary column packed with Monitor 5-μm 100-Å C18-modified silica from C18 reversed-phase column (Waters, Milford, MA) at a flow rate of 1 μl/min. The acylated peptides eluted during the second gradient. Each fraction was screened for molecular weight using MALDI-TOF mass spectrometry (Perspective Biosystems Model Voyager-Elite, Foster City, CA). Predicted mass values were calculated with the aid of Sherpa, a Macintosh-based protein and peptide analysis program, from the hlyA gene sequences for J96 (18) and pHly152 (16).

**Microcappillary HPLC Electrospray Ionization Tandem Mass Spectrometry—**Results from the initial screening indicated which HPLC fractions contained putative modification sites. Those fractions were analyzed by microbore HPLC coupled to a TSQ 7000 electrospray tandem quadrupole mass spectrometer (Finnigan, San Jose, CA). One μl of each Lys-C fraction was eluted from a 50-μm inner diameter × 12-cm capillary column packed with Monitor 5-μm 100-Å C18-modified silica from C18 reversed-phase column (Waters, Milford, MA) at a flow rate of 1 μl/min. The acylated peptides eluted during the second gradient. Each fraction was screened for molecular weight using MALDI-TOF mass spectrometry (Perspective Biosystems Model Voyager-Elite, Foster City, CA). Predicted mass values were calculated with the aid of Sherpa, a Macintosh-based protein and peptide analysis program, from the hlyA gene sequences for J96 (18) and pHly152 (16).

**Modification of Native Peptides and Synthesis of Acylpeptide Standards—**We treated the Lys-C and tryptic subdigest fractions containing acylated peptides with methanolic HCl to convert any carboxylate side chains, as well as C termini, to their methyl esters (22). This allowed unambiguous identification of Asp and Glu. Acetylation was employed to derivatize N termini and C termini, allowing easy differentiation between Lys and Gin, and y series and b series ions (23) in the CAD spectra. Synthetic standards, prepared using Fmoc (N-9-fluorenylmethoxycarbonyl) chemistry for both acylated and non-acylated peptides, were used to confirm the structures assigned by mass spectrometry. The details of the derivatization reactions and the synthetic chemistry have been described (24).

**Enzymatic Digestion with Lys-C—**Achromobacter protease I (EC 3.4.21.50, from Dr. T. Masaki, Ibaragi University) was used for all initial digests of HlyA. The toxin was digested at a ratio of 0.2 μg of enzyme per 1 nmol of substrate for 20–24 h at 37 °C. The digested products (~500 μl) were frozen at ~4 °C until fractionation by HPLC.
served by Ludwig et al. (7) when they attempted to digest their HlyA with trypsin or V8 protease.

**C14:0 Fatty Acylation—**HPLC fraction 39 from HlyAJ96 (Fig. 1A) contained two peptides that can be related to the predicted Lys-C fragments, with C14:0 acylation at Lys-690 (VLQEVKEEQEVS\_C14:0\_RTEK and EQEVSVGK\_C14:0\_RTEK), based on their molecular weights. Subdigestion of fraction 39 with trypsin and the MALDI-TOF MS results showed a peak at $m/z$ 1243 which corresponded to the product, EQEVSVGK\_C14:0\_R. CAD experiments showed a fragmentation spectrum (Fig. 2A) which yielded the expected y and b series ions. Virtually identical CAD fragmentation patterns were observed when a separate CAD experiment was performed using a C14:0 acylated synthetic peptide, thus confirming the C14:0 acylation at Lys-690 on HlyAJ96. This work was repeated, with the same results, for HlyApHly152. HPLC fraction 44 contained a Lys-C fragment with putative C14:0 acylation at Lys-564, FVTPLLTPGEIRERQGSK\_C14:0\_VEYITELLVK ($m/z$ 3777). This peptide was subdigested; CAD spectra were acquired, and the C14:0 modification was confirmed by synthesizing the tryptic fragment and analyzing the fatty acyl group itself by GC/MS. Again, the work was repeated for HlyA\_nHly152, with identical results. No modification at Lys-690 or Lys-564 was observed with the biologically inactive ProHlyAJ96 control.

**C15:0 and C17:0 Fatty Acylation—**Our initial goal was to identify the expected C14:0 acylation sites on HlyA, based on our interpretation of work by the Cambridge group (6) and Ludwig et al. (7). However, we found Lys-C HlyAJ96 fragments that eluted later than fractions 39 and 44 and that yielded $m/z$ values not predicted by theoretical peptide maps, allowing for acylation by common fatty acids such as C12:0, C14:0, C16:0, C18:0 or their unsaturated forms. Examples include peptides at $m/z$ 1615 and 2411 in fraction 42 (Fig. 1B) and $m/z$ 1643 and 2439 in fraction 41 (Fig. 1C), which are 14 and 42 mass units larger than the two C14:0-acylated Lys-690 peptides found in fraction 39 (Fig. 1A). Similarly, in fraction 45, two Lys-C fragments 14 and 42 mass units larger than the C14:0 acylated Lys-564 peptide in fraction 44 were also observed. We observed similar results for HlyA\_nHly152 but not for the inactive...
ProHlyAJ96 control. A number of potential modifications could give rise to these observations, based on molecular weight information alone, but subsequent analysis of these proteolytic fragments by CAD and electron impact GC/MS of the FAMEs clearly identified the fragments as C15:0 and C17:0 modifications of Lys-690 and Lys-564. Fig. 2 shows the CAD spectra of the C15:0-modified Lys-690 peptide (HPLC fraction 42) acquired following a tryptic subdigestion. The fragmentation pattern was identical to its C14:0 counterpart (Fig. 1), except that starting at the y2 and b8 ions, an increase of 14 mass units was noted. Similar CAD results were observed for the Lys-C fragments of HlyApHly152. We also converted the putative C15:0-acylated peptides in HPLC fraction 42 to methyl esters. Fig. 3 shows the expected mass increases from the original peptides (Fig. 1B) due to the addition of methyl ester groups to the C terminus and the aspartic acid residues. Tryptic subdigestion of these methyl ester derivatives also yielded a peptide with the expected CAD spectrum, suggesting a C15:0 acylation at Lys-690. We repeated these procedures for the remaining HPLC fractions, 41 and 45, which contained the putative C17:0 acylation at Lys-690 and both C15:0 and C17:0 at Lys-564. The MALDI-TOF and electrospray CAD data can successfully pinpoint the locations of these fatty acylations, but studies of the modifying groups themselves were necessary to confirm the conclusions suggested by the peptide CAD data. FAME analysis by capillary GC/MS provided both retention time data and the EI fragmentation patterns that taken together were unequivocal for the assignments of C15:0 (Fig. 4A) and C17:0 (Fig. 4B) as modifying groups.

Quantitative Analysis—Based on the capillary GC retention times for well characterized FAME standards, we identified the peaks corresponding to the C14:0, C15:0, and C17:0 modifications of HlyA. The amounts of each present, as calculated from the peak areas with background corrections, were 68% C14:0, 26% C15:0, and 6% C17:0, with a relative standard deviation of about 8%. This was in good agreement with our expectations based on the peptide electrospray data.

Biological Significance—Our results demonstrate that E. coli α-hemolysin from two sources with different genetic histories consists of a heterogeneous group of acylated proteins, as many as nine (two acylation sites and three possible modifying groups at each site). In contrast to the situation with CyaA described earlier, the data acquired over a 3-year period, involving many preparations of HlyApro, were remarkably consistent. The results for a single preparation of HlyApro were identical to those from HlyApro, within the expected limits of experimental error. No evidence of palmitoylation was observed in HlyA from either source.

Our data support the hypothesis that the substrate specificities of HlyC and CyaC (the acyltransferase from B. pertussis...
that activates CyaA) or their respective complexes with acyl-ACP are different. CyaC-mediated acylation of CyaA involves primarily palmitoylation (8, 9, 26) or unsaturated C-16 side chains. HlyC-mediated acylation of HlyA in a cell-free system was most efficient when C14:0 acyl-ACP was used as a substrate (2). Similarly, C14:0-modified HlyA produced in vitro was the most active form in the sheep red blood cell assay for hemolytic activity (5). Stanley et al. (2) and observations from recent biophysical studies with model membranes (27) have suggested that C14:0 side chains are not hydrophobic enough in themselves to allow HlyA to penetrate target cell membranes in a nonspecific manner, but they are sufficient to promote surface binding to target cell membranes. These observations may shed some light on the functional significance of the approximately 68% C14:0 in vivo acylation that we report here. However, the observations of C15:0 and C17:0 were unexpected and difficult to rationalize because these fatty acids are present in E. coli at concentrations too small to normally be considered significant. However, the cellular machinery responsible for HlyA activation is known to function with very low concentrations of acyl-ACP substrate (2). Odd-carbon fatty acids are not mentioned in authoritative reviews of chemical composition (28) and endogenous fatty acid metabolism (29) in E. coli, although they are known to occur in the membranes of Gram-negative bacteria generally (30) and in other taxonomic groups. However, C17:0 and C15:0 fatty acyl groups have not, to our knowledge, been reported as protein modifications, much less as substrates for the known internal lysine acylations (2). Therefore, the suggestion that their presence is simply a by-product of enzymes and (or) carrier proteins with loose specificity, whose primary end products are C14:0-modified lysine at Lys-564 and Lys-690, is probably not tenable. On the other hand, if HlyA were to achieve a better “fit” with C15:0 or C17:0 during a specific host cell protein interaction, e.g. a receptor, or between monomers in the putative oligomerization step some believe may be involved in pore formation, their presence could be rationalized. It will be interesting to examine the modification status of previously described HlyA mutants and HlyA/LktA hybrid proteins with altered target cell specificities (11, 31). Lally et al. (32) have suggested the β2 integrins as possible receptors for HlyA, but recent attempts to demonstrate β2 integrin-specific HlyA-mediated cytotoxicity have not been successful. Another possibility is that these side chains may promote interference with host cell signaling pathways by mimicking the action(s) of a lipid-containing host cell messenger. The fact that CyaA is acylated primarily with 16 carbon side chains whereas HlyA is acylated with 14, 15, and 17 carbon groups suggests that the two toxins differ significantly in the physical details of their interactions with target cell membranes.

Acknowledgments—We thank Ken Walsh and Lowell Ericsson for the use of their MALDI-TOF MS. We thank Houle Wang for construction of the modified Finnigian ESI interface on our TSQ 7000. David Goodlett at Bristol-Myers Squibb provided access to a Finnigian TSQ 7000, prior to delivery of our own instrument. We thank Marie Zhang for assistance with the toxin purification; James Kerwin and Peter Sebo for their advice and criticism; William Howald for the GC/MS work; Kerry Nugent for HPLC packing materials; and Jeanine M. Kanov for assistance with the manuscript.

REFERENCES

1. Welch, R. A., Bauer, M. E., Kent, A. D., Leeds, J. A., Moayeri, M., Regassa, L., and Swenson, D. L. (1995) Infect. Agents Dis. 4, 254–272
2. Stanley, P., Koronakis, V., and Hughes, C. (1998) Microbiol. Mol. Biol. Rev. 62, 309–333
3. Welch, R. A. (1991) Mol. Microbiol. 5, 521–528
4. Hess, J., Wels, W., Vogel, M., and Goebel, W. (1986) J. Bacteriol. 167, 16749–16755
5. Issartel, J. P., Koronakis, V., and Hughes, C. (1991) Nature 351, 759–761
6. Stanley, P., Packman, L. C., Koronakis, V., and Hughes, C. (1994) Science 266, 1059–1062
7. Ludwig, A., Garcia, F., Bauer, S., Jarchau, T., Benz, R., Hoppe, J., and Goebel, W. (1996) J. Bacteriol. 178, 5422–5430
8. Hackett, M., Guo, L., Shahanovitz, J., Hunt, D. F., and Hewlett, E. L. (1994) Science 266, 433–435
9. Hackett, M., Walker, C. B., Guo, L., Gray, M. C., Van, C. S., Ullmann, A., Shahanovitz, J., Hunt, D. F., Hewlett, E. L., and Sebo, P. (1995) J. Biol. Chem. 270, 20250–20253
10. Betsou, F., Sebo, P., and Guise, N. (1993) Infect. Immun. 61, 3583–3589
11. Pelletti, S., and Welch, R. A. (1996) Infect. Immun. 64, 3081–3087
12. Welch, R. A., Hull, R., and Falkow, S. (1983) Infect. Immun. 42, 178–186
13. Mayyeri, M., and Welch, R. A. (1997) Infect. Immun. 65, 2233–2239
14. Welch, R. A., Delinger, E. P., Minshaw, B., and Falkow, S. (1981) Nature 294, 665–667
15. Swenson, D. L., Bukanov, N. O., Berg, D. E., and Welch, R. A. (1996) Infect. Immun. 64, 3736–3743
16. Hess, J., Wels, W., Vogel, M., and Goebel, W. (1986) FEMS Microbiol. Lett. 34, 1–11
17. Wang, H., Lim, K. B., Lawrence, R. F., Howald, W. N., Taylor, J. A., Ericsson, L. H., Walsh, K. A., and Hackett, M. (1997) Anal. Biochem. 250, 162–168
18. Felmlee, T., Pelletti, S., and Welch, R. A. (1985) J. Bacteriol. 163, 94–105
19. Hunt, D. F., Alexander, J. E., McCormack, A. L., Martino, P. A., Michel, H., Shahanovitz, J., Sherman, N. E., Moseley, M. A., Jorgenson, J. W., and Tomer, K. B. (1991) in Techniques in Protein Chemistry II (Villarranca, J. J., ed.) pp. 441–454, Academic Press, New York
20. Shannon J. W., and Jorgenson, J. W. (1989) Anal. Chem. 61, 1128–1135
21. Moseley, M. A., Deterding, L. J., Tomer, K. B., and Jorgenson, J. W. (1991) Anal. Chem. 63, 1467–1473
22. Hunt, D. F., Yates, J. R., Shahanovitz, J., Winston, S., and Hauer, C. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6223–6227
23. Biemann, K. (1992) Annu. Rev. Biochem. 61, 977–1010
24. Gulden, P. H., Hackett, M., Addona, T., Guo, L., Walker, C. B., Sherman, N. E., Shahanovitz, J., Hewlett, E. L., and Hunt, D. F. (1996) in Mass Spectrometry in the Biological Sciences (Burlingame, A. L., and Carr, S. A., eds) pp. 281–306, Humana Press Inc., Totowa, NJ
25. Lepage, G., and Roy, C. C. (1986) J. Lipid Res. 27, 114–120
26. Basar, T., Huvlek, V., Benouskova, S., Halada, P., Hackett, M., and Sebo, P. (1999) J. Biol. Chem. 274, 10777–10783
27. Joseph, M., and Nagaraj, R. (1995) J. Biol. Chem. 270, 16749–16755
28. Neidhardt, F. C., and Umbarger, H. E. (1996) in Escherichia coli and Salmonella, Cellular and Molecular Biology (Neidhardt, F. C., et al., eds) pp. 13–16, American Society for Microbiology, Washington, D. C.
29. Cronan, J. E., and Rock, C. O. (1996) in Escherichia coli and Salmonella, Cellular and Molecular Biology (Neidhardt, F. C., et al., eds) pp. 612–636, American Society for Microbiology, Washington, D. C.
30. Kerwin, J. L. (1994) in Inosentoids and Other Natural Products, Evolution and Function (Nes, W. D., ed) pp. 163–201, American Chemical Society, Washington, D. C.
31. Forestier, C., Welch, C. (1991) Infect. Immun. 59, 4212–4220
32. Lally, E. T., Kieba, I. R., Sato, A, Green, C. L., Rosenbloom, J., Korostoff, J., Wang, J. F., Shenker, B. J., Orellano, S., Robinson, M. K., and Billings, P. C. (1997) J. Biol. Chem. 272, 30463–30469

6 R. A. Welch, manuscript in preparation.