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Sialyation of Haemophilus ducreyi lipooligosaccharides

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Sialylation of Haemophilus ducreyi Lipooligosaccharides:
Studies on the Cytidine 5'-Monophosphate N-acetylneuraminic Acid Synthetase

by

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DISSERTATION

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Collaborations

Chapters 2 and 3 are based largely on the following paper: Tullius, M. V., Munson, R. S., Jr., Wang, J. and Gibson, B. W. (1996). Purification, Cloning, and Expression of a Cytidine 5'-Monophosphate N-acetylneuraminic Acid Synthetase from *Haemophilus ducreyi*. *J. Biol. Chem. 271*(*26*), 15373-15380. The amino acid sequencing in Chapter 2 was performed by Lori Andrews in the laboratory of Al Burlingame at U.C.S.F. Dr. Anthony Campagnari (SUNY at Buffalo, NY) supplied several preparations of *H. ducreyi* strain 35000 for use in enzyme isolation. The cloning and DNA sequencing in Chapter 3 were performed by Jing Wang in the laboratory of Dr. Robert Munson in Columbus, Ohio. The construction of protein expression vectors was done by myself with the aid of Jing Wang and Dr. Munson in Dr. Munson's laboratory and at U.C.S.F. All other work was performed by myself at U.C.S.F.

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Chapter 5 is work being done in collaboration with Dr. Munson's laboratory. The construction of *H. ducreyi* isogenic mutants and and their complemented clones was performed by Jing Wang and Dr. Joel Bozue in Dr. Munson's laboratory. Jing also grew the *H. ducreyi* cultures used for LOS isolation. The isolation of LOS and its chemical and mass spectrometric characterization was performed by myself at U.C.S.F.

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ABSTRACT

Haemophilus ducreyi is a gram-negative bacterium causes the genital ulcer disease, chancroid. Previous studies have shown that the acidic monosaccharide N-acetylneuraminic acid (NeuAc, sialic acid) is found at high levels as the terminal sugar of the lipooligosaccharides (LOS) from the majority of *H. ducreyi* strains. As sialic acid is known to have important roles in cellular recognition and is a recognized virulence factor in several other pathogens it is believed that this sugar plays an important role in the pathogenesis of *H. ducreyi*.

To begin to elucidate the role of sialic acid, N-acetylneuraminic acid cytidylyltransferase (CMP-NeuAc synthetase) was isolated from a *H. ducreyi* cell extract. This enzyme catalyzes the reaction of CTP and NeuAc to form CMP-NeuAc, the nucleotide sugar donor used by sialyltransferases. Edman sequencing of the enzyme yielded 43 residues of sequence from which degenerate oligonucleotide probes were derived and used to clone the CMP-NeuAc synthetase gene (*neuA*) from a *H. ducreyi* genomic DNA library.

To characterize the enzyme, *neuA* was cloned into a T7 expression vector, the protein expressed in *Escherichia coli*, and purified to homogeneity (20 mg/L of culture). To identify residues important in binding the substrate CTP or involved in catalysis, chemical modification reagents were used to covalently modify and inactivate the CMP-NeuAc synthetase. Covalently labeled peptides were isolated and characterized by mass

spectrometry and Lysine-19, a residue conserved in CMP-NeuAc synthetases, was identified as the major site of modification.

Finally, a CMP-NeuAc synthetase deficient isogenic mutant of *H. ducreyi* was constructed. LOS from this isogenic mutant and a second mutant with an insertion in a putative sialyltransferase gene were isolated and characterized. The amount of NeuAc found as a component of the LOS was reduced to undetectable levels for the *neuA* mutant and to very low levels for the putative sialyltransferase mutant. Complementation of the defects was successful and proved the importance of these genes in sialylation. These mutants will enable the role of sialic acid in the pathogenesis of *H. ducreyi* to be determined.

List of Abbreviations

ACTH	adrenocorticotropic hormone
Anhydro-oC	4'5'-anhydro-2'3'-dialdehyde cytidine
Anhydro-ε-oC	4'5'-anhydro-2'3'-dialdehyde 3,N^4-etheno-cytidine
Ara-CTP	cytosine β-D-arabinofuranoside triphosphate
Bicine	N,N-bis(2-hydroxyetheyl)glycine
BSA	bovine serum albumin
ε-CTP	3,N^4-etheno-CTP
ESI-MS	electrospray ionization-mass spectrometry
Gal	galactose
Glc	glucose
GlcNAc	N-acetylglucosamine
Hep	heptose
HPLC	high performance liquid chromatography
IPTG	isopropyl b-D-thiogalactopyranoside
KDN	3-deoxy-D-glycero-D-galacto-nonulosonic acid
KDO	2-keto-3-deoxy-D-manno-octulosonic acid
LacNAc	N-acetyllactosamine
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MALDI-MS	matrix assisted laser desorption ionization-mass spectrometry
ManNAc	N-acetylmannosamine
MOPS	3-(N-morpholino)propane-sulfonic acid
α-MSH	α-melanocyte stimulating hormone
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycolylneuraminic acid
oAdo	2'3'-dialdehyde Adenosine
oADP	2'3'-dialdehyde ADP
oATP	2'3'-dialdehyde ATP
oCTP	2'3'-dialdehyde CTP
oUTP	2'3'-dialdehyde UTP
PEA	phosphoethanolamine
PEP	phosphoenolpyruvate
PGO	phenylglyoxal
PLP	pyridoxal 5'-phosphate
PSD	post source decay
PTH	phenylthiohydantoin
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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CHAPTER 1.

Overview of *Haemophilus ducreyi* Biology and Current Research

1.1 Chancroid and *Haemophilus ducreyi*

Chancroid, or soft chancre, is one of the five classical sexually transmitted diseases (Morse 1989). The incubation period is usually 4-7 days, but can range from 2 to 35 days (Morse 1989; Jessamine and Ronald 1990). The disease is characterized by painful genital ulcers, 2-3 mm deep, that are accompanied by inguinal lymphadenopathy in about half of chancroid patients. Infected lymph nodes can rupture to form ulcers, as well. Ulcers may also become infected with other organisms, leading to more extensive destruction of tissue (Morse 1989). The primary sites of ulceration are the skin and mucosal surfaces of the foreskin in males and the entrance of the vagina in females (Lagergard 1995). Other than involvement of inguinal lymph nodes, there is no systemic dissemination of chancroid, although autoinoculation of extragenital sites has been described (Trees and Morse 1995). The etiological agent was first isolated by Ducrey in 1889 by serial inoculation of the skin of patients with material extracted from their own genital ulcers (Morse 1989). The organism isolated, now known as *Haemophilus ducreyi*, is a gram-negative coccobacillus. Humans are the only known host of *H. ducreyi* and there does not appear to be significant, if any, asymptomatic carriage of the organism (Jessamine and Ronald 1990). Small breaks in the epithelium that may occur during sexual activity are thought to be necessary for the organism to enter the host and cause infection (Morse 1989). Bacterial toxins and the host response to the disease are both likely to play roles in the subsequent tissue destruction. Both a humoral and cell mediated immune response occurs in chancroid patients, however, it appears to be non-protective as re-infection can occur (Lagergard 1995).

1.2 Classification

Bergey's Manual of Systematic Bacteriology classifies members of the genus *Haemophilus*, in the family *Pasteurellaceae*, as gram-negative, non-motile, coccobacilli that have a requirement for X factor (hemin) and/or V factor (NAD or NADP), reduce nitrates, and have a guanine-plus-cytosine content of 37-44% in their DNA. (Kilian and Biberstein 1984). As expected from belonging to this group, *H. ducreyi* has been shown to have a requirement for hemin, reduces nitrate, and has a guanine-plus-cytosine content of 38-39% (Kilian 1976; Casin et al. 1985). In addition, antibodies to antigens from *H. ducreyi* are cross-reactive with antigens from other members of the family *Pasteurellaceae* (Alfa et al. 1992). However, several lines of evidence suggest that *H. ducreyi* should be assigned to a separate genus in the family *Pasteurellaceae.* Casin et al. showed that the *H. ducreyi* type strain, CIP 542, was only 0-6% related to other *Haemophilus* species using DNA hybridization (Casin et al. 1985). Furthermore, *H. ducreyi* strains from diverse geographical locations were relatively homogeneous, with DNA relatedness of 80-100% to the type strain. The results of genetic transformation and rRNA-DNA hybridization studies on the family *Pasteurellaceae* also support the reclassification of *H. ducreyi* (Albritton et al. 1986; De Ley et al. 1990). *H. ducreyi* has been found to have a different quinone content from other *Haemophilus* species, which also questions the current classification of *H. ducreyi* (Carlone et al. 1988).

1.3 Prevalence

Chancroid is rather common in Africa and Asia. Patients presenting with genital ulcer disease (GUD) account for 18-70% of all sexually transmitted disease (STD) cases and one third to one half of these are diagnosed as chancroid (Morse 1989). Most of the remaining GUD cases are caused by herpes simplex virus or *Treponema pallidum* (the causative agent of syphilis). In the United States, the situation is quite different with

GUD patients comprising only a small percentage of STD patients. In addition, most GUDs are due to syphilis or herpes, not chancroid (Jessamine and Ronald 1990). However, there have been a number of outbreaks in the United States in recent years. From 1965 to 1984 the number of reported cases of chancroid in the United States was usually less than 1000 (mean, 925) (Trees and Morse 1995). A sharp increase led to a high of 4986 cases in 1987, which steadily declined to 1399 cases in 1993 (Trees and Morse 1995). The prevalence of chancroid in the Unites States may be largely underreported, however (Schulte et al. 1992). At the present time, diagnosis of chancroid requires culture of *H. ducreyi* from a clinical specimen. However, culture only has a sensitivity of 53-84% and many clinics in the United States are not even equipped to distinguish chancroid from syphilis and herpes. In fact, a survey of 115 health departments in the United States and Puerto Rico conducted by the Centers for Disease Control and Prevention (CDC) found that only 14% were able to perform culture for *H. ducreyi* (Schulte et al. 1992). Recent studies have shown that chancroid may be a common cause of GUD in some cities. Using a new polymerase chain reaction (PCR) diagnostic assay, 51% of patients with GUD at a Jackson, Mississippi STD clinic were infected with *H. ducreyi* (Webb et al. 1995). Likewise, in New Orleans, Louisiana, 39% of patients with non-syphilitic GUD were culture-positive for *H. ducreyi* and the authors believed that many of the culture-negative patients also had chancroid based on a PCR assay and other observations (DiCarlo et al. 1995). Chancroid has a high male to female ratio in the United States, about 10 or 20 to 1, and has been associated with prostitution, drug use, and the exchange of sex for drugs (Blackmore et al. 1985; Flood et al. 1993; DiCarlo et al. 1995).

1.4 Antibiotic Resistance

H. ducreyi has acquired extensive plasmid and chromosomally mediated antimicrobial resistance. Many drugs once effective against chancroid can no longer be used because of high levels of antimicrobial resistance. Resistance to sulfonamides, trimethoprim, trimethoprim-sulfamethoxazole, aminoglycosides, tetracycline, chloramphenicol, penicillin and other beta-lactams, and amoxicillin-clavulanic acid has been documented (Morse 1989; Knapp et al. 1993; Van Dyck et al. 1994). However, several drug classes: macrolides, expanded spectrum cephalosporins, and quinolones, are still quite effective (Aldridge et al. 1993). Erythromycin, azithromycin, ceftriaxone, and ciprofloxacin have all been used successfully against *H. ducreyi* . However, there have been some recent problems with single dose failure with ceftriaxone and ciprofloxacin and there is concern about resistance to quinolones developing in *H. ducreyi* as it has in some other bacteria (Plourde et al. 1992; Tyndall et al. 1993; Tyndall et al. 1994). A strain resistant to ciprofloxacin was isolated recently in Thailand (Knapp et al. 1993). A single intramuscular dose of ceftriaxone, a single dose of azithromycin orally, or erythromycin four times a day orally for seven days are the regimens currently recommended by the CDC (Trees and Morse 1995).

1.5 Immune Response

H. ducreyi infection elicits both a humoral and cellular immune response. IgG, IgM, and IgA antibodies are produced by chancroid patients against *H. ducreyi* antigens (Roggen et al. 1994). After ulceration, antibody levels rise over 4 weeks (Roggen et al. 1994; Chen et al. 1997). The response is primarily IgG, although, some patients have been found to have high IgM levels as well (Schalla et al. 1986; Alfa et al. 1992; Alfa et al. 1993; Roggen et al. 1994). This agrees with a recent study which demonstrated that the humoral immune response is highly variable among individuals in contrast to the

situation observed in syphilis patients (Brown et al. 1995). Alfa et al. have found that IgG levels to outer membrane proteins are stable for at least 8 months, but the IgM response to lipooligosaccharides declined to approx. 20% after 2 months (Alfa et al. 1993). A humoral immune response may not be elicited until the ulcerative stage of disease because no antibodies were detected in the experimental human model of infection in which subjects are infected for up to 2 weeks, but without ulceration (Spinola et al. 1996). It is believed that the antibodies produced are not protective because of the reinfection and autoinoculation that can occur in chancroid patients (Lagergard 1995).

The cellular immune response to *H. ducreyi* infection has not been characterized as well as the humoral immune response. Two recent analyses of biopsies of chancroid ulcers revealed a dense inflammatory infiltrate which contained primarily macrophages and T lymphocytes, with only a few plasma cells (King et al. 1996; Magro et al. 1996). These results are consistent with a cell-mediated immune response. Similarly, biopsies of pre-ulcer pustules from experimentally infected subjects contained neutrophils, T lymphocytes, and macrophages (Spinola et al. 1996). The swine model has also produced similar results (Hobbs et al. 1995). However, these findings are somewhat at odds with the classical descriptions of chancroid ulcers in which a plasma cell rich infiltrate is observed, indicative of a humoral immune response (Sheldon and Heyman 1946; Freinkel 1987). The reason for these differences have not been determined. Other studies that suggest a cell-mediated immune response occurs during chancroid have shown elevated levels of soluble interleukin-2 receptors in chancroid patients' serum and urine, and suppression of interleukin-2 production in human mononuclear lymphocytes with chancroid patients plasma (Abeck et al. 1990; Korting et al. 1993). Furthermore, a recent study has also shown that *H. ducreyi* expresses antigens that stimulate T lymphocytes in vitro (Van Laer et al. 1995). Protective immunity in the temperature dependent rabbit model has been shown to occur through cell-mediated mechanisms,

however, its relevance to the human immune response is not known (Desjardins et al. 1992).

1.6 Genital Ulcer Disease (GUD) and the Human Immunodeficiency Virus (HIV)

There has been an increased interest in chancroid and *H. ducreyi* in recent years because an association has been found between GUDs and HIV seropositive status (Jessamine and Ronald 1990; Wasserheit 1992). Several studies on heterosexual Africans have demonstrated a 4-5 fold increased risk of being HIV positive in chancroid, and other GUD patients, after accounting for behavioral variables such as the number of sexual partners and amount of contact with prostitutes (Wasserheit 1992). It has been suggested that disruption of the protective epithelial surfaces in GUD patients allows for easier transmission of HIV. Also, the increased presence of HIV susceptible T lymphocytes and macrophages at the infection site may increase the chance of an HIV negative person with GUD acquiring the virus. Likewise, an HIV positive person with GUD may have a greater chance of passing the virus to a partner because of greater amounts of HIV infected cells at ulcer sites (Wasserheit 1992). Another cause for concern has been reports describing increased treatment failure in some instances with HIV positive chancroid patients (MacDonald et al. 1989; Ballard et al. 1996).

1.7 Recent Advances in *H. ducreyi* Research

There have been many advances in *H. ducreyi* research recently, many of them occurring over the course of this research project. There are now several useful animal models to use in assessing virulence, in vitro models to determine adhesion and invasion properties of strains, genetic tools for generating isogenic mutants, advances in diagnostic assays, and identification of several potential virulence factors.

1.7.1 Animal Models

H. ducreyi is a strict human pathogen and studies of virulence had been hampered by lack of an appropriate animal model for many years. Although intradermal injection of rabbits was developed as a model to test virulence, it suffered from many drawbacks that will be discussed below. In recent years, significant progress has been made in developing more relevant animal models to study the pathogenesis of *H. ducreyi.*

1.7.1.1 Experimental Human Model

The experimental human model of infection by *H. ducreyi* described by Spinola and colleagues is perhaps the most relevant model available for chancroid, however, there are several limitations in its use (Spinola et al. 1994; Spinola et al. 1996). Volunteers were inoculated on their arms using an allergy testing device with pins that break the integrity of the epithelium, delivering bacteria loaded on the tines (prongs) to the epidermis and dermis. This method of inoculation, through abraded skin, should be a much better approximation of the natural route of infection as compared to intradermal injection (used in some other animal models) which bypasses the upper layers of the skin entirely. Furthermore, inoculation of only 1500 to 35000 colony forming units (CFU) caused lesions in the majority of subjects. Because the device only delivers approx. 0.1% of the solution loaded on its tines, the authors estimated that as few as 1-35 CFU was capable of causing infection. The infection was allowed to proceed for up to 2 weeks or until a pustule developed, at which point the experiment was stopped to prevent ulceration. Viable *H. ducreyi* were recovered for up to 1 week after inoculation from the surface of lesions, indicating that the bacteria were being shed from these pre-ulcer lesions. Recovery of greater numbers of *H. ducreyi* from lesion biopsies than were initially inoculated demonstrated that the bacteria were replicating in vivo. Some limitations of the model are: 1) genital and mucosal surfaces cannot be infected, 2) ulceration and lymphadenopathy cannot be observed, and 3) antibiotic resistant bacteria

cannot be used. However, the model is reported to be very similar to the initial stages of the natural disease and should be useful in studying virulence factors involved in the initial stages of *H. ducreyi* pathogenesis in its natural host. A humoral immune response was not detected even after 2 weeks of infection and no protective immunity was observed in initial rechallenge studies.

1.7.1.2 Temperature Dependent Rabbit Model

Rabbits have been used as an animal model of *H. ducreyi* lesion formation for many decades, however, very large numbers of organisms (at least 10^8 CFU) were required to produce lesions (Feiner et al. 1945; Hammond et al. 1978). A substantial improvement to the model was made by housing the rabbits at 15-17°C in order to lower their skin temperature (Purcell et al. 1991). Because *H. ducreyi* requires temperatures less than 37°C (optimal, approx. 33°C) and rabbits have a high core temperature of approx. 39°C, the authors hypothesized that *H. ducreyi* would not grow well in rabbit skin. In rabbits housed at 15-17°C, reproducible production of lesions was achieved with inocula of 10^5 CFU injected intradermally into the back, with as few as 10^3 CFU producing lesions in some instances. Viable, replicating bacteria were required for lesion formation. Heat killed bacteria did not produce lesions and rabbits pretreated with an antibiotic effective against *H. ducreyi* did not develop lesions when injected with live bacteria. Viable *H. ducreyi* could be recovered from lesions for almost 2 weeks after inoculation. One disadvantage of the model is the method of inoculation, intradermal injection, which is quite different from natural infection. Also, although the lesions share some similarities with human ulcers, they are not identical. A humoral immune response to *H. ducreyi* infection was demonstrated. Further studies have shown both humoral and cell-mediated immune responses to *H. ducreyi* infection and to challenge with *H. ducreyi* antigens (Hansen et al. 1994; Desjardins et al. 1995; Desjardins et al. 1996). Partial

protective immunity was obtained in these studies and was determined to be due to a cell-mediated immune response.

1.7.1.3 Swine Model

Inoculation of *H. ducreyi* through abrasions in the epithelium of pig ears and backs is a promising, new model of *H. ducreyi* infection (Hobbs et al. 1995). A fairly large inocula of 10^7 CFU was used, however, an allergy testing device was used to deliver the inocula (as in the experimental human model) so that the infecting dose may have been as little as 10^4 CFU. This is an order of magnitude less than the dose used in the temperature dependent rabbit model (Purcell et al. 1991), but is far greater than the doses used in the human model (Spinola et al. 1996). The authors stated that lower doses will be used in future studies to determine the minimal infective dose. Lesion formation was reported to be similar to both natural chancroid and the lesions formed in the human model. Heat killed bacteria did cause some inflammation, but did not produce ulcers. Viable *H. ducreyi* was recovered from lesions up to 17 days after inoculation of pig ears and 11 days after inoculation of pig backs. Increased antibodies to *H. ducreyi* antigens were detected in infected pigs, but in contrast to the temperature dependent rabbit model, no protective immunity was observed when pigs were rechallenged. This is similar to findings in the human model (Spinola et al. 1996). The authors pointed out a number of advantages to this model: 1) more relevant route of inoculation as compared to intradermal inoculation used in the rabbit and primate models, 2) lower costs than for primates, 3) housing at room temperature, 4) pure bred pigs are available, possibly making analysis of immune responses simpler, 5) reagents for characterizing cellular immune response are readily available, 6) many inocula can be made on the large surface area of the pigs backs and ears, and 7) antibiotic resistant strains can be used and ulcers allowed to form in contrast to experimental human infections.

1.7.1.4 Primate Models

Two primate models have been described, one using intradermal inoculation into the abdominal area of adult male *Macaca mulatta* monkeys (Sturm 1997) and the other intradermal inoculation of the foreskin or labia of adult pigtailed macaques (*Macaca nemestrina*) (Totten et al. 1994). In the study involving inoculation of genital areas, lesions developed that resembled human disease only in the males. Also, large inocula of 10^7-10^8 CFU were used, which are much greater than the inocula used in other models. However, the minimal dose and whether injection was necessary were not determined in this study, so further experiments may define this better. Other disadvantages of the model are the high cost of the animals, minimal number of injections possible per animal, and the lack of response in the females. Viable organisms were required for ulcer formation and organisms could be recovered from lesions for up to 20 days. Antibodies to *H. ducreyi* antigens were detected after one week of infection. Inguinal lymphadenopathy was observed, making this the only model in which this process can be studied. Using an animal more similar to humans, the authors state that there is a greater chance of identifying virulence factors that are only produced under conditions found in the host. This model will probably find limited use, with most virulence testing being done in the rabbit, human, and swine models.

The minimal ulcerative dose was only 10^5 CFU in the primate model using intradermal inoculation of the abdominal wall (Sturm 1997). This dose was decreased further if iron was available either by pretreating the monkeys with iron or including it in the bacterial inocula. A very interesting finding was that the *H. ducreyi* type strain, CIP 542, which is avirulent in the rabbit (Purcell et al. 1991) and swine models (Hobbs et al. 1995) was able to cause ulcers in this model. This strain produced an ulcer in a person accidentally infected in a laboratory experiment (Trees et al. 1992), so this primate model may be a better measure of virulence than the rabbit and swine models.

1.7.1.5 Mouse Model

Intradermal and/or subcutaneous injection of 10^7 CFU of *H. ducreyi* was capable of producing lesions in mice that closely resembled human chancroid ulcers (Tuffrey et al. 1988). However, further study revealed that viable bacteria were not necessary for lesion formation (Tuffrey et al. 1990). Heat killed organisms also produced lesions and mice pretreated with an antibiotic that kills *H. ducreyi* developed lesions as well. Furthermore, purified lipooligosaccharides from *H. ducreyi* and *Neisseria gonorrhoeae* also produced ulcers, demonstrating the toxicity of lipooligosaccharides, but revealing the model has limited use for studying *H. ducreyi* pathogenesis.

1.7.1.6 Mouse Subcutaneous Chamber Model

In contrast to the mouse skin model, *H. ducreyi* was able to grow when inoculated into subcutaneous chambers implanted in mice (Trees et al. 1991). The advantage of this model is the long infections that are maintained, most lasting longer than 2 months. The model may prove useful for identifying changes in antigen expression during in vivo growth and in finding virulence factors that are upregulated or only expressed in vivo. However, the infection is very artificial compared to human chancroid and cannot be used to address important issues such as adhesion, invasion, and ulcer formation. No further results using this model have been reported in the six years since it was first described.

1.7.2 Tissue Culture Models

A number of studies investigating the ability of *H. ducreyi* to adhere to and invade eukaryotic cells have been published in the past five years. There is some difficulty in comparing results of the various studies because more often than not different cell lines, *H. ducreyi* strains, and/or experimental methods were used. However, it is clear that *H. ducreyi* adheres tightly to several different cell lines, some of which are quite relevant to

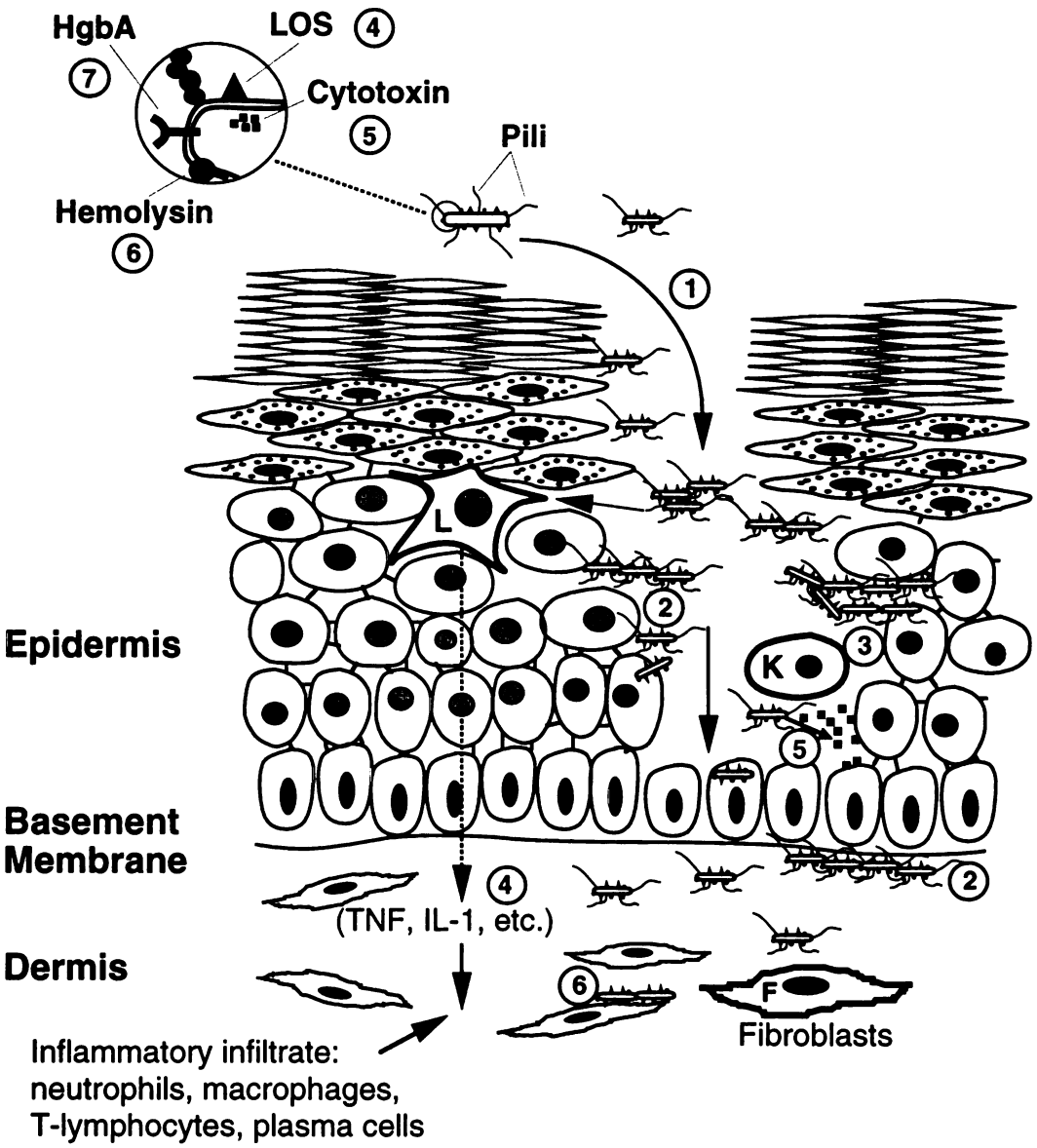
the natural infection, and can probably invade some cell types in certain situations. Shah et al. demonstrated *H. ducreyi* adherence to and internalization into four epithelial cell lines (Shah et al. 1992). The most extensive internalization occurred with HeLa cells (human cervix epithelial cell line) and was found to be a function of both the bacterial and eukaryotic cells. *H. ducreyi* was found to adhere to 3 cell lines of genital origin by Lammel and coworkers (Lammel et al. 1993). Invasion was extensive with a human foreskin fibroblast cell line (HFF), less with an endometrial adenocarcinoma cell line, and in contrast to the findings of Shah et al., no invasion occurred with HeLa cells. Often HeLa and HEp-2 (human larynx epithelial cell line) cell lines are used to study the invasiveness of bacteria (Finlay and Falkow 1988), however, the foreskin fibroblast should be a more relevant model for *H. ducreyi* pathogenesis because the organism is almost certain to encounter this cell type during infection. Lagergard et al. found substantial adherence of 10 different *H. ducreyi* strains to HEp-2 and HeLa cell lines (Lagergard et al. 1993). Only minimal invasion was detected and the authors speculated that it might be an in vitro artifact due to the high bacterial inoculum used in the study. *H. ducreyi* was found to adhere to foreskin fibroblast cultures derived from both adults and children, with only minimal internalization (Alfa et al. 1993; Alfa and DeGagne 1994). A further study by the same group, found that gentamicin-killed bacteria were able to adhere as well as viable bacteria, suggesting that the necessary adhesins are constitutively expressed on the bacterial surface (Alfa and DeGagne 1994). In a study involving 34 strains of *H. ducreyi*, the vast majority (28) adhered strongly to HFF cells (Alfa et al. 1995). Those strains that were defective in adherence were also found to be avirulent in the temperature dependent rabbit model. Although foreskin fibroblasts are a relevant cell type for *H. ducreyi* adherence studies, foreskin epithelial cells and keratinocytes are cell types that are likely to be encountered earlier in the course of infection (Brentjens et al. 1994; Totten et al. 1994). Brentjans et al. tested several *H. ducreyi* strains and found that they all adhered extensively to keratinocytes derived from

neonatal foreskins. Totten et al. demonstrated tight adherence of 6 out of 8 *H. ducreyi* strains to foreskin epithelial cells and HEp-2 cells. The two strains that adhered weakly were avirulent in the temperature dependent rabbit model. A moderate level of invasion was also found for both of these cell lines, 10-20 times greater than a non-invasive *Escherichia coli* strain, but 5-6 times less than a very invasive *Salmonella* strain. Whether intracellular growth is important for *H. ducreyi* pathogenesis is uncertain at this time, but an interesting observation was made recently which suggests that it may. Large numbers of *H. ducreyi* were found filling cells presumed to be granulocytes in analysis of clinical smears (Ahmed et al. 1995). However, it is not known whether the bacteria in the cell were still viable.

1.7.3 Virulence Factors

Pathogenicity refers to the ability of a microorganism to cause disease in its animal or plant host (Smith 1977). Pathogenicity is multifactorial, generally requiring a pathogen to: 1) enter and multiply within the host, 2) evade or disrupt the host's defense mechanisms, and 3) cause damage to the host (Smith 1977). Many virulence factors can be involved in each of these areas. Non-virulent or less virulent strains may still express virulence factors, just not enough of them, or the right combination of factors, to cause significant damage to the host. Likewise, virulent strains may not possess all the virulence factors identified for its species, but still cause disease (Smith 1977). *H. ducreyi* is likely to possess several of the virulence factors discussed below, and perhaps others that have not yet been identified, in order to cause disease. However, at present, the exact roles of many of these factors in *H. ducreyi* pathogenesis are largely unknown. Figure 1.1 is a model of *H. ducreyi* infectivity that illustrates the possible role of some of these virulence factors which will be discussed in the following sections.

Fig. 1.1. **Model of *H. ducreyi* infectivity.** (1) *H. ducreyi* is thought to enter the host through small breaks in the integrity of the skin leading to the formation of an ulcer which extends into the dermis. An infiltration of neutrophils, macrophages, T lymphocytes, and plasma cells is commonly observed. (2) Colonization may be greatly aided by the organism's ability to adhere to cells such as keratinocytes (K) and fibroblasts (F) and the extracellular matrix proteins found in the basement membrane. Pili and LOS may play key roles in adhesion. (3) Microcolony formation on host cell surfaces may also be a virulence factor. (4) LOS likely causes tissue damage by stimulating macrophages (including skin macrophages, Langerhans cells (L)) and other cells to release high levels of tumor necrosis factor (TNF), interleukin-1 (IL-1), and other cytokines. (5) A diffusable cytotoxin and a cell associated hemolysin (6) may also be involved in tissue destruction. (7) A hemoglobin receptor (HgbA) allows the organism to obtain a suitable source of iron from the host.


HgbA
7
LOS
4
Cytotoxin
5
Pili
Hemolysin
6
1
L
2
3
K
5
Epidermis
Basement Membrane
4
(TNF, IL-1, etc.)
2
Dermis
6
F
Fibroblasts
Inflammatory infiltrate:
neutrophils, macrophages,
T-lymphocytes, plasma cells

1.7.3.1 Resistance to Serum and Phagocytic Killing

Resistance to the bactericidal activity of serum and phagocytic killing has been correlated with virulence in a number of bacteria, and was one of the first virulence factors identified in *H. ducreyi* (Odumeru et al. 1984; Joiner 1985). Although *H. ducreyi* strains were classified as virulent or avirulent using the old rabbit model in this study, at least one of the virulent strains (strain 35000) is virulent in the temperature dependent rabbit model and two of the avirulent strains (A76 and A77) are avirulent in the model, so the results are likely to still be reliable. Complement was necessary for serum killing and neutrophil phagocytosis (Odumeru et al. 1984; Odumeru et al. 1985). The classical pathway of complement was found to be the predominant mode of serum killing. A latter study by different authors confirmed these results, but also showed that a significant amount of neutrophil phagocytosis could occur even in the absence of complement (Lagergard et al. 1995). These authors also showed that IgG, IgM, and complement factors were deposited on the *H. ducreyi* outer membrane from non-immune normal human serum (NHS). Some of the strains tested were highly resistant to serum killing, and none were completely killed in 60% NHS, conditions that completely killed a control *H. influenzae* strain.

1.7.3.2 Adherence and Microcolony Formation

In order for a pathogen to enter and multiply within a host it must often first adhere to host mucosal surfaces (Beachey 1981). Often a correlation between adherence in tissue culture models and infectivity of the organism is observed. The interaction of bacteria with eukaryotic cell surfaces involves both non-specific and specific recognition events. With regard to specific recognition, a number of pathogens have been shown to produce protein adhesin molecules on their surface that bind to receptors on host cell surfaces. These receptors are very often carbohydrate structures (Beachey 1981).

As mentioned in the section on tissue culture models, *H. ducreyi* is capable of adhering to several types of cell lines including cells that the organism would contact in the course of infection. *H. ducreyi* forms cohesive colonies and chains or clumps of organisms are observed in gram stains of culture grown organisms (Albritton 1989; Morse 1989). Organisms growing together as chains or microcolonies on the surface of cells in tissue culture models have also been observed (Alfa 1992; Lagergard et al. 1993; Lammel et al. 1993; Alfa et al. 1995). Furthermore, Gram stains of ulcer exudates often contain clusters or chains of organisms as well (Eichmann 1996). Alfa et al. have proposed that this microcolony formation may be a virulence factor of *H. ducreyi*, as strains deficient in adherence and microcolony formation were less virulent in the temperature dependent rabbit model (Alfa et al. 1995). The microcolonies may help to prevent phagocytosis of the organisms. Totten et al. had also noted previously that two strains that were avirulent in the temperature dependent rabbit model adhered poorly in a tissue culture model and did not form chains when grown in broth culture (Totten et al. 1994). A recent study has suggested that low levels of GroEL prevents formation of long chains of *H. ducreyi* in a tissue culture model (Parsons et al. 1997). Two of these strains are avirulent in the temperature dependent rabbit model and the third has not been tested.

A study of 21 *H. ducreyi* isolates has demonstrated a high degree of binding to the extracellular matrix (ECM) proteins, fibrinogen, fibronectin, collagen, gelatin, and laminin (Abeck et al. 1992). Nineteen of the isolates bound to 4 or 5 of the 5 ECM proteins tested, one isolate bound to three, and one only to two of the proteins. These ECM proteins are important components of the basement membrane between the epidermis and dermis layers of the skin (Odland 1991). Since *H. ducreyi* ulcers penetrate into the dermis, it is quite likely that contact with these proteins occurs during infection and binding to them may aid in colonization and subsequent ulcer formation.

1.7.3.3 Lipooligosaccharides (LOS)

As this research project has focused on an enzyme involved in LOS biosynthesis in *H. ducreyi*, the role of LOS will be covered in more detail in a separate section. Only those studies directly related to LOS as a virulence factor of *H. ducreyi* will be discussed here. Shortly after Odumeru et al. demonstrated that serum resistance was a virulence factor for *H. ducreyi*, two additional studies by the same group found that LOS was an important factor in serum resistance and was a virulence factor as well (Odumeru et al. 1984; Odumeru et al. 1985; Odumeru et al. 1987). One study showed that LOS from serum sensitive strains could be used to remove the bactericidal activity of NHS by prior absorption of the serum with LOS (Odumeru et al. 1985). In contrast, absorption of serum with the LOS from serum resistant strains did not reduce its bactericidal activity. The authors believed that removal of natural antibodies in the non-immune NHS was responsible for the loss of bactericidal activity, as the majority of the complement activity remained after treatment. Antibodies that cross react with *H. ducreyi* antigens in non-immune sera are well known and presumably arise from contact with commensals or prior infection with *Haemophilus* species (Alfa et al. 1992). Further study of the LOS revealed differences between the LOS of virulent and avirulent strains (Odumeru et al. 1987). The LOS of avirulent strains produced somewhat different electrophoretic profiles and had a lower ratio of total sugar to KDO, suggesting a smaller oligosaccharide component. Campagnari et al. found that *H. ducreyi* LOS was capable of causing lesions in rabbits that resembled those produced by live and heat killed organisms (Campagnari et al. 1991). Interestingly, the LOS of *N. gonorrhoeae* produced similar size lesions, while the LOS from *H. influenzae* and the lipopolysaccharide from *E. coli* produced much smaller lesions.

1.7.3.4 Hemolysin

H. ducreyi possesses a cell associated hemolysin related to the *Proteous mirabilis* and *Serratia marcesans* pore-forming family of hemolysins (Palmer and Munson 1995; Totten et al. 1995). The target of the hemolysin in vivo is not known. The hemolysin may act on erythrocytes to release hemoglobin for growth, it may damage epithelial cells contributing to ulcer formation, or it may destroy macrophages, lymphocytes, and neutrophils in order to blunt the host immune response (Palmer and Munson 1995; Stevens et al. 1996). The hemolysin has been shown to be the factor responsible for the activity of a contact cytotoxin that damages human foreskin fibroblasts described previously (Alfa 1992; Alfa et al. 1996; Palmer et al. 1996). Results of animal and human challenge studies with an isogenic mutant lacking hemolytic activity have not been published yet, but should clarify the role of the hemolysin in *H. ducreyi* pathogenesis.

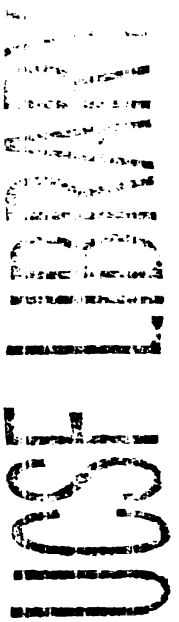
1.7.3.5 Diffusable Cytotoxin

H. ducreyi also expresses a secreted cytotoxin that does not damage fibroblasts, but is extremely toxic to HeLa and HEp-2 epithelial cell lines (Purven and Lagergard 1992). The cytotoxin has recently been shown to be a homolog of the *E. coli* cytolethal distending toxin (CDT), however, the in vivo role of this toxin in *H. ducreyi* or *E. coli* pathogenesis is not known (Cope et al. 1997). A study conducted on 100 strains of *H. ducreyi* from diverse geographic locations found that 89% of the strains produced high levels of the cytotoxin activity, suggesting that it may be an important virulence factor (Purven et al. 1995).

1.7.3.6 Pili

Pili are a type of adhesin that are often found on the outer-membrane of gram-negative bacteria (Beachey 1981). Spinola and coworkers have identified piliated *H.*

ducreyi cells, isolated the pilin monomer, and cloned its gene (Spinola et al. 1990; Brentjens et al. 1996). Pili was recovered from 12 out of 12 strains examined, but piliated cells were only detected in 10 of the strains. The pili are described as fine and tangled. They comprise a novel class of pili, not sharing homology with other known pilin proteins (Brentjens et al. 1996). The role of pili in *H. ducreyi* pathogenesis is not known, however one study showed that only non-piliated strains failed to bind the extracellular matrix protein laminin (Abeck et al. 1992). Humans with chancroid and rabbits injected with whole bacteria both produce antibodies to pili (Frisk et al. 1995). An isogenic mutant that does not express pili has been constructed and will allow a more definitive analysis of the biological role of *H. ducreyi* pili (Brentjens et al. 1996).



1.7.3.7 Outer Membrane Proteins (OMPs)

Investigators are interested in outer-membrane proteins (OMPs) for their possible role in pathogenesis, but also as potential vaccine candidates.

1.7.3.7.1 OmpA

The major OMP (MOMP) of *H. ducreyi* is an *E. coli* OmpA homolog, and not a classical porin protein that are usually the MOMPs in other gram-negative bacteria (Spinola et al. 1993). OmpA may have a role in gram-negative and gram-positive bacteria and probably functions to stabilize the structure of the outer membrane (De Mot and Vanderleyden 1994). OmpA appears to have some role in virulence as well, because an *E. coli* OmpA-negative ($OmpA^-$) mutant was found to be more susceptible to serum killing than the wild type strain (Weiser and Gotschlich 1991). Furthermore, OmpA was also shown to enhance the invasion of *E. coli* into brain microvascular endothelial cells, which is an important event in meningitis caused by *E. coli* (Prasadarao et al. 1996). A second OmpA homolog (OmpA2) was detected in a *H. ducreyi* isogenic mutant defective in expression of MOMP (Klesney-Tait et al. 1997). Construction of an OmpA2-negative

strain and a double mutant (OmpA2$^-$, MOMP$^-$) will be very useful in determining the role of these proteins in *H. ducreyi* pathogenesis.

1.7.3.7.2 PAL

Another conserved OMP under investigation is a protein with homology to *E. coli* PAL (peptidoglycan-associated lipoprotein) (Spinola et al. 1992; Spinola et al. 1996). It probably functions to stabilize the outer membrane as with OmpA (De Mot and Vanderleyden 1994). *H. ducreyi* PAL has several qualities that are desirable for a vaccine candidate, such as being conserved in *H. ducreyi* strains, exposed on the surface, and antigenically stable (Spinola et al. 1996). Both chancroid patients and healthy subjects produced antibodies to *H. ducreyi* PAL. The antibodies were not *H. ducreyi* specific as they could be remove by adsorption with lysates from other *Haemophilus* species, and unfortunately, the antibodies from normal human sera were not bactericidal. It remains to be determined if antibodies from chancroid patients or raised against purified PAL are bactericidal.

1.7.3.8 Iron Acquisition

Nearly all bacteria require iron for growth (Otto et al. 1992). Because the level of free iron in the human host is extremely low (approx. 10^{-18} M), pathogens have evolved several means for acquiring iron from the host (Bullen 1981; Otto et al. 1992). *H. ducreyi* has a strict requirement for exogenous hemin and cannot synthesize porphyrins or porphobilinogens when supplied with their precursor, delta-aminolevulinic acid (Hammond et al. 1978). *H. ducreyi* can use hemin, hemoglobin, hemoglobin complexed with haptoglobin, heme complexed with albumin, or catalase as a source of iron, but not cytochrome c, lactoferrin, transferrin, or $FeCl_3$ (Lee 1991).

1.7.3.8.1 Hemoglobin Binding Receptor

H. ducreyi expresses a hemoglobin receptor on its outer membrane that is regulated by heme concentration, but not iron levels (Elkins 1995). An isogenic mutant lacking the hemoglobin receptor was no longer able to use hemoglobin as a source of heme (Elkins et al. 1995). A similar isogenic mutant constructed by another group of investigators was found to be less virulent in the temperature dependent rabbit model (Stevens et al. 1996). In addition to causing a much lower degree of ulceration, the isogenic mutant was not recovered from any of the lesions, suggesting that it was not capable of replicating in the rabbit host. This report is quite significant because it is the first instance of an isogenic mutant of *H. ducreyi* showing reduced virulence in an animal model.

1.7.3.8.2 Iron Regulated Proteins

In addition to the hemoglobin receptor, a number of other proteins have been found to be upregulated under low heme and iron conditions (Abeck et al. 1990; Lee 1991). However, no functions have been assigned to any of these other proteins.

1.7.3.8.3 *fur* Homolog

H. ducreyi also possesses a functional *fur* (ferric uptake regulation) system (Carson et al. 1996). In the presence of iron, the *fur* gene product can block transcription of *fur*-regulated genes. The role of *fur* in *H. ducreyi* pathogenesis is unknown.

1.7.3.9 Heat Shock Proteins (HSPs)

H. ducreyi expresses four heat shock proteins (HSPs) (Brown et al. 1993). One of these, GroEL, is expressed at high levels in vitro, with basal levels up to 5 times greater than those found in *E. coli* (Parsons et al. 1997). GroEL is involved in folding and stabilization of proteins under various stress conditions, such as changes in temperature,

exposure to oxidative conditions, or contact with eukaryotic cells. A *H. ducreyi* clone with a multicopy plasmid expressing DnaK, a presumed negative modulator of GroEL, produced 2.3 fold less GroEL than the parent strain and was much less capable of surviving heat and oxidative stress (Parsons et al. 1997). Parsons et al. discovered that GroEL may have a vital role in the adherence of *H. ducreyi* to eukaryotic cells (Parsons et al. 1997). First, the level of GroEL produced by *H. ducreyi* was shown to double in an in vitro adherence assay. Also, the strain carrying the DnaK plasmid, along with two other *H. ducreyi* strains that naturally produced lower levels of GroEL, could not form adherent chains in a cell culture model. These two strains are also avirulent in the temperature dependent rabbit model. Antibodies to GroEL are produced by both chancroid patients and experimentally infected animals (Brown et al. 1993; Parsons et al. 1997).

1.7.4 Genetic Tools

It wasn't until 1992 that a system for constructing isogenic mutants of *H. ducreyi* was developed (Hansen et al. 1992). Hansen and coworkers used electroporation to introduce linear and circular DNA into *H. ducreyi*. Furthermore, they demonstrated that homologous recombination of mutated genes occurred readily, allowing for the isolation of isogenic mutants with disrupted genes. This has been quite a significant advance in the study of *H. ducreyi* pathogenesis, but as pointed out by the authors, the method requires cloning of *H. ducreyi* genes into *E. coli* for genetic manipulation and some genes may be difficult or impossible to clone. Systems using transposons on plasmids that cannot replicate in *H. ducreyi* were developed independently by two groups to allow for the mutagenesis of *H. ducreyi* genes in *H. ducreyi* (Palmer and Munson 1995; Stevens et al. 1995). Demonstrating the utility of these techniques, in a short time several studies have been published in which isogenic mutants have been constructed which lack potential virulence factors. Genes coding for LOS biosynthetic enzymes (Stevens et al.

1995), hemolysin (Palmer and Munson 1995; Totten et al. 1995), hemoglobin receptor (Elkins et al. 1995; Stevens et al. 1996), MOMP (Klesney-Tait et al. 1997), and pili (Brentjens et al. 1996) have been targeted with these methods. As will be discussed in Chapter 5, as part of a collaborative project, we have successfully created isogenic mutants using similar methods that lack functional CMP-NeuAc synthetase and sialyltransferase genes and are unable to produce NeuAc containing LOS.

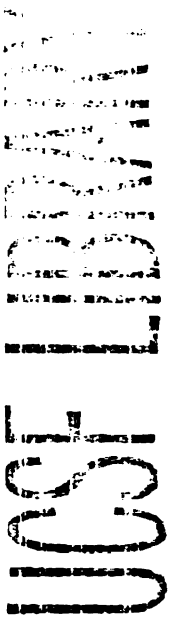
1.7.5 Diagnosis

Diagnosis of chancroid by culture of *H. ducreyi* from genital lesions has been the standard by which other diagnostic methods have been judged. Because of the fastidious nature of *H. ducreyi*, isolation rates are generally low, ranging from 53-84% (Morse 1989), with the higher rates being obtained in endemic areas and in labs with more experience culturing *H. ducreyi*. Furthermore, proper media is often not available in clinics to culture *H. ducreyi*, and diagnosis is based upon clinical presentation and treatment outcome (Schulte et al. 1992; Eichmann 1996). Clinical diagnosis can be very unreliable because of frequent atypical presentation of lesions and mixed infections (Sturm et al. 1987). Direct gram-staining of smears of clinical specimens is also an insensitive method of diagnosis (Sturm et al. 1987). There has been a great deal of interest in developing a simple, rapid, inexpensive, and sensitive diagnostic assay for chancroid. With this as a goal, assays that detect *H. ducreyi* DNA, antigens, and antibodies to *H. ducreyi* antigens have been developed.

1.7.5.1 DNA Based Diagnostic Assays

The first DNA based diagnostic assays used radiolabeled oligonucleotide probes to detect *H. ducreyi* DNA (Parsons et al. 1989; Rossau et al. 1991). These studies demonstrated that a specific response to *H. ducreyi* DNA could be obtained. However, the detection levels were probably not low enough to be useful for clinical samples and

required the use of radioactivity. A polymerase chain reaction (PCR) assay was first developed by Chui et al. and was found to be very sensitive for *H. ducreyi*, detecting 51 out of 51 strains from six different continents (Chui et al. 1993). Furthermore, the assay was very specific, not reacting with 12 species of other bacteria, including members of the *Pasteurellaceae*. As few as 1 CFU could be detected and the assay worked well on clinical samples, however, the procedure was rather time-intensive and also required radioactivity. Other similar PCR assays have been developed (Johnson et al. 1994; Johnson et al. 1995; Parsons et al. 1995), but a much simplified PCR assay was introduced by West et al. that required much less sample processing and used colorimetric detection that could be read by eye (West et al. 1995). A similar PCR assay was developed as part of a multiplex PCR (M-PCR) assay that detects the DNA of the three most common causes of genital ulcer disease, *H. ducreyi*, *T. pallidum*, and herpes simplex virus (Orle et al. 1996). The assay requires very minimal specimen preparation and also uses colorimetric detection. The assay was highly sensitive (98.4%) and specific (99.6%) for *H. ducreyi*, as well as for the other infectious agents. Culture sensitivity was only 74.2% for *H. ducreyi* by comparison. Because of its high specificity and sensitivity, along with its ability to detect all three of the major causes of GUD simultaneously, the M-PCR assay promises to be a very useful tool for diagnosing GUD. All of these PCR assays detected *H. ducreyi* DNA in a significant percentage of culture negative cases, pointing out the inadequacy of culture in diagnosing chancroid.



1.7.5.2 Serologic Diagnostic Assays

A number of assays have been developed for the detection of *H. ducreyi* antigens, either protein or LOS, using rabbit polyclonal antibodies or mouse MAbs. Detection has been by indirect immunoflouresence (Denys et al. 1978; Slootmans et al. 1985; Schalla et al. 1986; Karim et al. 1989; Finn et al. 1990; Ahmed et al. 1995), enzyme linked immunosorbent assay (ELISA) (Roggen et al. 1993; Ahmed et al. 1995), or imunolimulus

method (Hansen et al. 1995). Antibodies raised to *H. ducreyi* can be cross-reactive with antigens of other *Haemophilus* species and some other bacteria as well. Removing this cross-reactivity is important in order to obtain an assay of high specificity, but doing so may decrease sensitivity. Most investigators using rabbit polyclonal antibodies have tried to remove this cross-reactivity by first adsorbing the serum with antigens from various *Haemophilus* and other bacterial species (Denys et al. 1978; Slootmans et al. 1985; Roggen et al. 1993) although other investigators have found that cross-reactivity could be largely removed by sufficient dilution of the antiserum (Schalla et al. 1986; Finn et al. 1990). Only a few of these studies have tested the assay on clinical specimens (Schalla et al. 1986; Karim et al. 1989; Roggen et al. 1993; Ahmed et al. 1995). The specificity and sensitivity of an ELISA assay were 100%, but the study involved only a limited (30) set of clinical samples (Roggen et al. 1993). The sensitivity and specificity of an indirect immunoflouresence assay was not as good as a PCR assay, but was better than culture (Ahmed et al. 1995).

A similar number of studies have investigated the possibility of detecting antibodies to *H. ducreyi* antigens in patients sera as a means of diagnosing chancroid (Schalla et al. 1986; Museyi et al. 1988; Alfa et al. 1992; Desjardins et al. 1992; Alfa et al. 1993; Duncan et al. 1994; Roggen et al. 1994; Brown et al. 1995; Chen et al. 1997; Rakwar et al. 1997). As with antigen detection, there is the problem of cross-reactive antibodies. In addition, some studies have also found high levels of antibodies reactive with *H. ducreyi* antigens even after adsorbing the sera with other *Haemophilus* species (Alfa et al. 1992; Roggen et al. 1994). Many studies have used only a single strain of *H. ducreyi* to prepare antigens for the capture of the patients anti-*H. ducreyi* antibodies, however, a combination of antigens from a diverse selection of *H. ducreyi* strains is probably a better method (Roggen et al. 1994; Brown et al. 1995). Along with these problems, antibody detection will not be a useful diagnostic method for reasons that were not elucidated until recently. First, the IgG response to *H. ducreyi* infection lasts for at

least 8 months (Alfa et al. 1993) and probably much longer (Rakwar et al. 1997), so that both past and present *H. ducreyi* infections are being detected by these assays. Second, the sensitivity of the assays are greatest when sera is collected at 4 weeks or longer after ulceration (Desjardins et al. 1992; Chen et al. 1997). Finally, a recent study showed relatively poor performance compared to M-PCR (Chen et al. 1997). However, these assays may be quite useful for epidemiological studies measuring the prevalence, both past and present, of *H. ducreyi* infection in a community.

1.7.6 Strain Typing

A typing scheme for *H. ducreyi* would be a very useful epidemiological tool allowing studies on geographic distribution and prevalence of strains and whether some strains are more pathogenic than others. Several methods for typing *H. ducreyi* strains have been investigated, including: 1) enzyme profiles (Van Dyck and Piot 1987), 2) serologically by immunotyping (Slootmans et al. 1985) or by immunoprofile (Roggen et al. 1992), 3) OMP profiles (Odumeru et al. 1983; Taylor et al. 1985), and 4) ribotyping (Sarafian et al. 1991; Brown and Ison 1993; Pillay et al. 1996). *H. ducreyi* is a relatively homogeneous species (Casin et al. 1985), and most typing methods have had little success. Van Dyck et al. found that 200 strains of *H. ducreyi*, isolated from 4 continents, that were tested with 95 different substrates yielded only 3 different enzyme profiles (Van Dyck and Piot 1987). Both OMP and immunoblot profiles were also quite limited even though a fairly large number of strains were studied (Odumeru et al. 1983; Taylor et al. 1985; Roggen et al. 1992). Ribotyping, which is based on the agarose gel pattern of restriction digests of ribosomal DNA, may prove to be a useful typing system. An initial study found 8 different ribotypes in only 14 isolates collected in a one month period in Kenya (Sarafian et al. 1991). Brown and Ison improved on this by developing a non-radioactive ribotyping method (Brown and Ison 1993). The authors distinguished 16 ribotypes out of 30 strains from geographically diverse locations. A more recent study

identified 13 different ribotypes from only 30 isolates collected from South Africa (Pillay et al. 1996). It seems likely that more ribotypes will be found in larger studies. This method appears to have a high degree of resolution, but it usefulness in analyzing a large number of geographically diverse strains remains to be determined.

1.8 The Role of Carbohydrates in Cellular Recognition

It is now recognized that carbohydrates, as components of glycolipids and glycoproteins of the cell membrane, are often involved in important events in cell recognition (Sharon and Lis 1993). Carbohydrates are involved in the attachment of eukaryotic cells to one other, and the binding of bacteria, viruses, hormones, and toxins to eukaryotic cells. An often cited example of carbohydrate mediated cell-cell adhesion is leukocyte extravasation which occurs as part of the inflammation process, and requires selectin proteins on endothelial cells and leukocytes to recognize carbohydrate ligands on the opposing cell (Feizi 1993). One of the best studied areas of carbohydrate mediated adhesion involves the binding of adhesin proteins from microorganisms to carbohydrate ligands (usually referred to as receptors in this situation) on host cells (Beachey 1981; Karlsson 1989; Sharon and Lis 1993). A good example of the specificity of these interactions is the binding of *E. coli* strains expressing type 1 fimbriae to epithelial cells of the urinary tract. Expression of type 1 fimbriae allows *E. coli* to bind mannose containing carbohydrates. When mice infected with these mannose specific *E. coli* were given a mannose derivative or antibodies to block mannose binding sites, colonization was greatly reduced compared to control mice (Beachey 1981; Sharon and Lis 1993).

The reverse situation, in which carbohydrate ligands on the bacteria bind to protein receptors on the host cell membrane has been less well characterized, however, interactions of this latter type have been reported as well. Recently it was shown that the carbohydrate from a *Chlamydia trachomatis* glycoprotein is involved in attachment of the

organism to HeLa cells (Swanson and Kuo 1994). Lipopolysaccharides are usually the most abundant carbohydrate on the outer membrane of gram-negative bacteria and some recent studies have shown that these molecules can be involved in adhesion of bacteria to host cells (Porat et al. 1995; Porat et al. 1995).

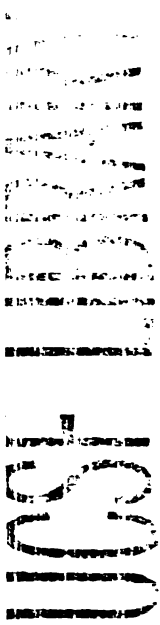
1.9 Lipopolysaccharides (LPS) and Lipooligosaccharides (LOS)

The outer membrane of gram-negative bacteria is composed of phospholipids, proteins, lipopolysaccharides (LPS), and sometimes other types of polysaccharides (Nikaido and Vaara 1985). The membrane is thought to be asymmetric, with LPS on the outer surface and phospholipids making up the inner surface (Nikaido and Vaara 1985). The structure of LPS along with the biochemistry and genetics of LPS biosynthesis in enteric organisms such as *Salmonella typhimurium* and *E. coli* has been extensively studied (Hitchcock et al. 1986; Raetz 1990; Raetz 1993; Schnaitman and Klena 1993).

LPS is the agent responsible for the long recognized toxicity of endotoxin preparations from gram-negative bacteria (Freudenberg and Galanos 1990). Although the terms endotoxin and LPS are often used synonymously, LPS should refer only to a purified material while endotoxin is a complex of LPS and outer membrane proteins (Hitchcock et al. 1986; Burrell 1994). Normally, humans are only exposed to endotoxin, not LPS. Large amounts of endotoxin in the blood stream that may occur during severe gram-negative infections can cause fever, hypotension, leukopenia, tachycardia, disseminated intravascular coagulation, and multiorgan failure (Schletter et al. 1995). The mortality rate for the resulting endotoxic shock is 20-50%. This toxicity is due to a stimulation of host cells by endotoxin to secrete high levels of mediators such as interleukin-1 (IL-1), IL-6, tumor necrosis factor (TNF), platelet activating factor, and thromboxane A_2 (Kielian and Blecha 1995; Schletter et al. 1995). Monocytes and

macrophages are the cells most activated by endotoxin, but other cell types are affected as well (Schletter et al. 1995).

LPS from enteric organisms is composed of the hydrophobic lipid A moiety that forms the outer membrane, a core of 9-11 sugars, and a variable number of O-antigen repeating units where each unit consists of 1-7 sugars (Hitchcock et al. 1986; Raetz 1993) (Fig. 1.2). The core region is fairly conserved while the O-antigens are quite variable among strains. This variability is observed in the over 1000 immunochemical variants of O-antigen found in *Salmonella* species (Raetz 1990). LPS preparations comprise a heterogeneous population of molecular species and typically 5-10% of the LPS in a preparation will lack O-antigen entirely (Freudenberg and Galanos 1990). The designation rough-LPS refers to LPS from mutants that lack O-antigen completely and is based on the typical colony morphology of such mutants which appears rough compared to the smooth appearance of wild-type strains (Hitchcock et al. 1986). For the most part, the genes for the biosynthesis of the core and O-antigen are organized into contiguous clusters, with somewhat less clustering of the lipid A biosynthesis genes (Schnaitman and Klena 1993). Most of the gene products are believed to be soluble or bound to the inner cytoplasmic membrane (Schnaitman and Klena 1993).



Using both isolated and chemically synthesized lipid A, it has been shown that the lipid A portion of LPS is the minimal structure required for the toxic properties of endotoxin (Raetz 1993). It is now known that soluble serum proteins and membrane receptors bind to the lipid A moiety of LPS specifically and with high affinity and are involved in the host response to endotoxin (Kielian and Blecha 1995; Schletter et al. 1995; Viriyakosol and Kirkland 1995). Although great progress has been made in determining the role of these proteins in binding LPS and activating cells, the complex set of events that lead to the eventual release of mediators are not fully understood (Schletter et al. 1995). Certain features of the lipid A structure are essential for toxicity and it should be noted that not all lipid A's are toxic. For example, the LPS from the

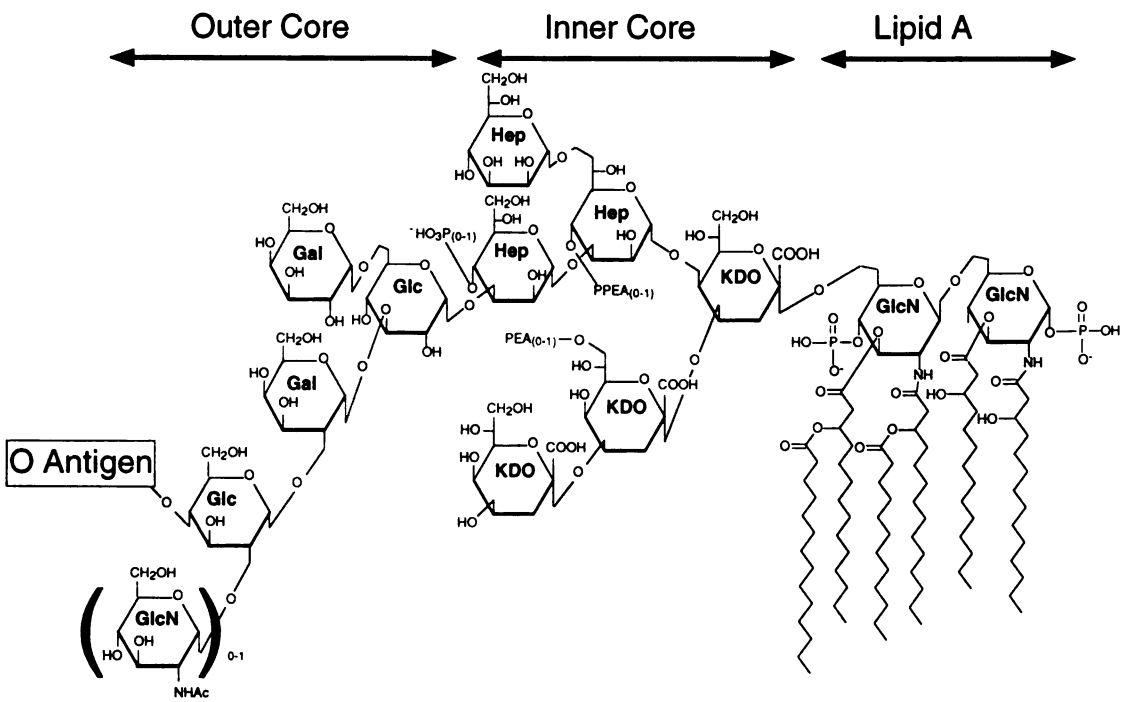


Fig. 1.2. **Structure of *E. coli* LPS.** LPS is composed of a Lipid A, a core oligosaccharide, and O-antigen repeating units. The core oligosaccharide is often described in terms of a highly conserved inner core and a more variable outer core region. However, the greatest variability is in the different O-antigen repeating units that strains express.

intracellular pathogen *Chlamydia trachomatis* is much less potent than *S. minnesota* and *N. gonorrhoeae* LPS (Ingalls et al. 1995). Also, the nitrogen-fixing bacterium *Rhizobium leguminosarum* produces LPS that is not toxic, as does the photosynthetic bacterium *Rhodobacter sphaeroides*, whose LPS is actually an antagonist of some of the effects of *E. coli* lipid A (Raetz 1993; Brozek et al. 1996; Jarvis et al. 1997).

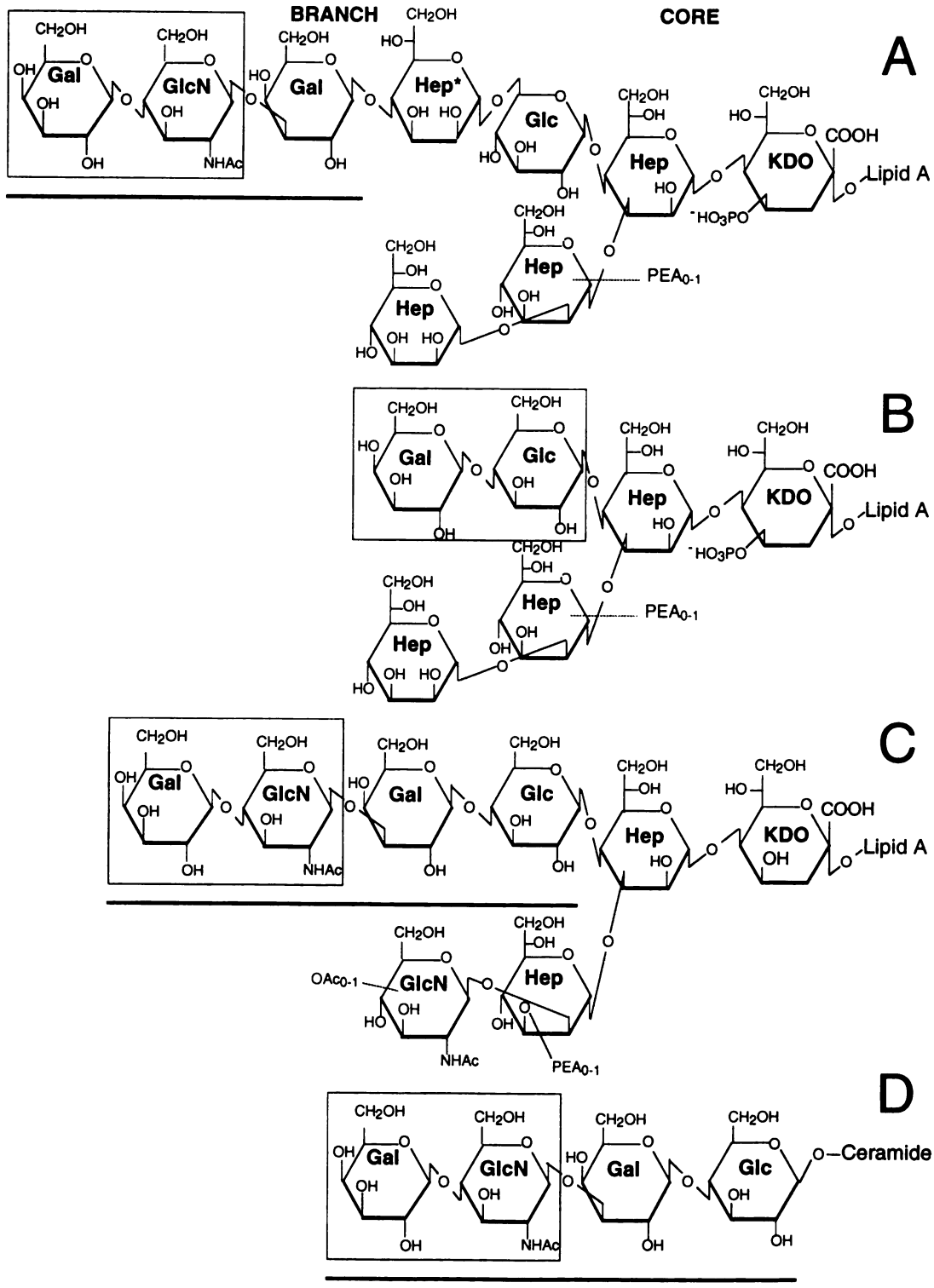
Although the LPS from non-enteric organisms has not been studied as extensively as LPS from enteric organisms, it is now known that many non-enteric pathogens produce LPS that lacks O-antigen (Hitchcock et al. 1986). In addition to *H. ducreyi*, other non-enteric pathogens such as *Haemophilus influenzae*, *N. gonorrhoeae*, *Neisseria meningitidis*, and *Bordetella pertussis* have been shown to produce "rough-type" LPS (Jennings et al. 1980; Inzana 1983; Peppler 1984; Odumeru et al. 1985; Griffiss et al. 1988). LPS is often a virulence factor in pathogens and as with the rough-LPS from *H. ducreyi*, the rough-LPS from *H. influenzae*, *N. gonorrhoeae* , *N. meningitidis* have also been identified as virulence factors (Weiser et al. 1990; van Putten and Robertson 1995). At first this discovery was quite surprising because *Salmonella* mutants that lose the ability to produce O-antigen become avirulent (Hitchcock et al. 1986). The rough-LPS of non-enteric organisms is often referred to as lipooligosaccharide (LOS), which seems appropriate considering the size of the carbohydrate chain and that these organisms never produce the longer chain LPS with O-antigen. The oligosaccharide region appears to be more heterogeneous than the core region of LPS from enteric organisms and may be playing a role similar to the variable O-antigen in the LPS from enteric organisms (Hitchcock et al. 1986). Although still not as well characterized as enteric LPS biosynthesis, much has been discovered about the genetics of LOS biosynthesis in *H. influenzae* and *Neisseria* spp. in recent years (Gotschlich 1994; Jennings et al. 1995; van Putten and Robertson 1995; Hood et al. 1996; Wakarchuk et al. 1996). The only *H. ducreyi* LOS biosynthetic genes identified and cloned so far have been the CMP-NeuAc

synthetase gene (as part of this project), the putative sialyltransferase gene (see Chapter 5), and two others arranged in tandem (Stevens et al. 1997).

The structure of the LOS from a number of *H. ducreyi* strains has been determined recently, primarily by work done in our lab and one other (Melaugh et al. 1992; Gibson et al. 1993; Campagnari et al. 1994; Melaugh et al. 1994; Schweda et al. 1994; Schweda et al. 1995; Ahmed et al. 1997; Gibson et al. 1997). In addition, LOS structures from *N. meningitidis* (Jennings et al. 1980; Dell et al. 1990; Michon et al. 1990; Gamian et al. 1992; Pavliak et al. 1993; Kim et al. 1994; Wakarchuk et al. 1996; Kogan et al. 1997), *N. gonorrhoeae* (Takayama et al. 1986; Yamasaki et al. 1988; Gibson et al. 1989; John et al. 1991; Yamasaki et al. 1991; Yamasaki et al. 1991; Kerwood et al. 1992; Gibson et al. 1993; Yamasaki et al. 1994), and *H. influenzae* (Helander et al. 1988; Phillips et al. 1990; Phillips et al. 1992; Gibson et al. 1993; Phillips et al. 1993; Schweda et al. 1993; Lee et al. 1995; Schweda et al. 1995; Phillips et al. 1996; Masoud et al. 1997; Risberg et al. 1997) have been characterized by our group and others. Most strains of *H. ducreyi* examined to date produce a heterogeneous mixture of LOS glycoforms. However, nearly all of them synthesize a LOS species with a nine sugar oligosaccharide moiety as the major species. A smaller LOS species with six sugars has been found to be the predominant species in a few strains. Figure 1.3 summarizes the structures of the major LOS species isolated from *H. ducreyi* along with a related LOS structure from *Neisseria* species. Because *Neisseria* species produce LOS structures similar to those of *H. ducreyi* and the role of sialic acid in their pathogenesis has been the subject of many recent studies (see section 1.10.6), these organisms (and the LOS they produce) serve as useful models for investigating the role of LOS and sialic acid in the pathogenesis of *H. ducreyi.*

A key discovery was made by Mandrell et al. when they found that the LOS from *N. gonorrhoeae* and *N. meningitidis* were immunochemically similar to antigens on human blood cells (Mandrell et al. 1988). Previously, Jennings et al. had determined the

Fig. 1.3. **Structures of the major LOS species identified in *H. ducreyi* strains and a related Neisserial LOS structure.** (A) Although LOS preparations are usually quite heterogeneous, this structure is found in the vast majority of *H. ducreyi* strains tested to date and is the predominant species identified. Up to approximately half of (A) contains NeuAc attached to the terminal galactose (Gal) residue. A small percentage of the LOS species may have an additional GlcNAc or Galβ1-4GlcNAc attached to the terminal Gal of (A) instead of NeuAc. Also, truncated species lacking the terminal Gal, Galβ1-4GlcNAc, and Galβ1-4GlcNAcβ1-3Gal are sometimes observed in lesser quantities. The heptose marked with an asterisk is D-glycero-D-mannoheptose which differs in configuration from the L-glycero-D-mannoheptose residues of the core. (B) This structure (or one with an additional GlcNAc linked to the terminal Gal) is the predominant LOS species in a few *H. ducreyi* strains. These strains lack higher molecular weight LOS species such as (A). (C) An LOS species found in some *N. gonorrhoeae* and *N. meningitidis* strains that is quite similar to (A) (see (Mandrell and Apicella 1993) for review). This species is also capable of being sialylated on the terminal Gal. (D) Structure of paragloboside. Ceramide is the lipid component of GSLs. Galβ1-4GlcNAc (N-acetyllactosamine, LacNAc) is boxed and the epitope believed to be recognized by the MAb 3F11 is underlined in all the structures. There are some small differences in the fatty acid composition of the lipid A of *H. ducreyi* and *Neisseria* spp. compared to *E. coli* lipid A, but otherwise the structures are very similar to the one shown in Fig. 1.2. PEA, phosphoethanolamine.


BRANCH
CORE
A
B
C
D
Gal
GlcN
Hep*
Glc
Hep
KDO
Lipid A
COOH
NHAc
PEA0-1
OAc0-1
-HO3PO
Ceramide

structure of the oligosaccharide from the major LOS species from several *N. meningitidis* strains (Jennings et al. 1983). The non-reducing terminal tetrasaccharide is identical to the oligosaccharide portion of the human glycosphingolipid (GSL), paragloboside, which is a precursor for human blood group antigens (Hakomori 1981; Mandrell et al. 1988). The antibody used by Mandrell and coworkers, designated 3F11, was originally raised to the LOS of *N. gonorrhoae*, and was found to agglutinate human erythrocytes and bind to isolated paragloboside and the related Ii antigens (Mandrell et al. 1988). In addition to being present in erythrocytes, Ii antigens are found in lymphocytes, monocytes, and neutrophils (Dunstan 1986) and paragloboside is a major constituent of the neutral GSLs from neutrophils (Macher and Klock 1980; Fukuda et al. 1985). The 3F11 epitope is found in the vast majority of *H. ducreyi* and *N. gonorrhoeae* strains tested to date, along with many *N. meningitidis* and *H. influenzae* strains (Mandrell et al. 1988; Campagnari et al. 1990; Mandrell et al. 1992).

This mimicry of host structures may contribute to pathogenesis by allowing the bacteria to evade the host's immune response or establish infection by adhering to host cells (Mandrell et al. 1992). It is quite likely that the host will be unable to raise an antibody response to these LOS structures as it is known that normal human sera does not contain functional antibodies against paragloboside (Kaise et al. 1985). Several other LOS structures that mimic different human GSLs can also be found in strains of *N. gonorrhoeae*, *N. meningitidis*, and *H. influenzae* (Mandrell and Apicella 1993). These pathogens are also capable of undergoing phase and antigenic variation of their LOS, both in vitro and in vivo, and this is believed to be a major factor in their successful evasion of the host immune response (Weiser et al. 1989; Roche and Moxon 1995; van Putten and Robertson 1995). In contrast to these organisms, the structural studies mentioned previously, along with electrophoretic, and immunochemical analysis of the LOS of *H. ducreyi* reveals that the LOS does not seem to undergo phase variation (Ahmed et al. 1995; Hansen et al. 1995; Ahmed et al. 1997). Also, so far the only

structure found to mimic human GSLs is the one expressing the 3F11 epitope (Campagnari et al. 1990; Ahmed et al. 1995; Ahmed et al. 1997).

Host mimicry is being found in other pathogens as well and may play an important role in the diseases these organisms cause. For example, the LPS of the pathogens *Campylobacter jejuni* and *Helicobacter pylori* has been found to mimic host structures (Moran et al. 1996; Salloway et al. 1996). Also, the GSLs of *Trypanosoma cruzi* were determined to be similar to the GSLs of heart muscle cells (Vermelho et al. 1997). Antibodies to these cross-reactive epitopes may be important in the pathogenesis of Guillain-Barre syndrome (*C. jejuni*) and Chagas disease (*T. cruzi*) (Moran et al. 1996; Vermelho et al. 1997).

Another very exciting discovery was finding N-acetylneuraminic acid (NeuAc) as a component of the LOS of several bacterial pathogens. The next section will discuss some of the important roles this sugar has in cellular recognition and pathogenesis. Studies by our group and others have shown that the LOS from *H. ducreyi*, *H. influenzae*, *N. gonorrhoeae*, and *N. meningitidis* contain NeuAc attached to the galactose of the terminal N-acetyllactosamine (LacNAc) disaccharide (Mandrell et al. 1990; Mandrell et al. 1991; Mandrell et al. 1992; Gibson et al. 1993; Melaugh et al. 1994; Melaugh et al. 1996). An earlier study had actually detected the presence of NeuAc in a hydrolysate of *H. ducreyi* LOS, however, no structural information was obtained (Odumeru et al. 1987).

1.10 Sialic Acid

1.10.1 Biochemistry

NeuAc is a nine carbon, α-keto acid, monosaccharide that normally occurs as the terminal residue of carbohydrates from cell surface glycolipids and glycoproteins (Schauer et al. 1995). NeuAc is one of nearly 40 N-acylated or deaminated neuraminic acid derivatives that have been identified in biological samples (Schauer et al. 1995;

Kelm et al. 1996). This amount of diversity is quite striking in a monosaccharide and there are no other sugars in which this type of diversity is known. This family of sugars are referred to as sialic acids, although often the general term sialic acid is used even when referring only to NeuAc. I will use the general terms sialic acid, and sialic acids, when discussing the general properties of these sugars and NeuAc only when the need to distinguish this sugar from the other sialic acids arises. The N-acyl group is either acetyl or glycolyl, one or more hydroxyls may be acetylated, and phosphate, sulfate, lactate, and methyl groups have been found on hydroxyls as well (Schauer et al. 1995). Sialic acid is usually found linked to galactose (Gal), N-acetylgalactosamine (GalNAc), or another molecule of sialic acid. Although commonly found in higher animals and all mammals, sialic acids have been found in relatively few microorganisms and only NeuAc, acetylated derivatives of NeuAc, and deaminated neuraminic acid derivatives (KDN), have been detected in those bacteria containing sialic acid (Schauer et al. 1995; Troy II 1995). Because of its terminal position on carbohydrates, sialic acid is one of the first molecules encountered in cellular interactions and has been found to have important roles in cellular recognition (Schauer et al. 1995).

1.10.2 Biosynthesis of Sialic Acid Containing Glycoconjugates

The biosynthesis of sialic acid containing glycoconjugates is similar in bacteria and mammals, although there are some differences. The pathway for the synthesis of sialic acid containing LOS is shown in Figure 1.4 and Figure 1.5 shows the mammalian biosynthetic pathway of sialic acid containing glycolipids and glycoproteins. In bacteria, NeuAc is synthesized by the condensation of N-acetyl mannosamine (ManNAc) and phosphoenol pyruvate (PEP) catalyzed by NeuAc synthetase. NeuAc synthetase was first purified, although not to homogeneity, from *N. meningitidis* (Warren and Blacklow 1962). It was not until this year that the enzyme was purified to homogeneity from *E. coli* K1 (Vann et al. 1997). The genes coding for these two enzymes are homologous and

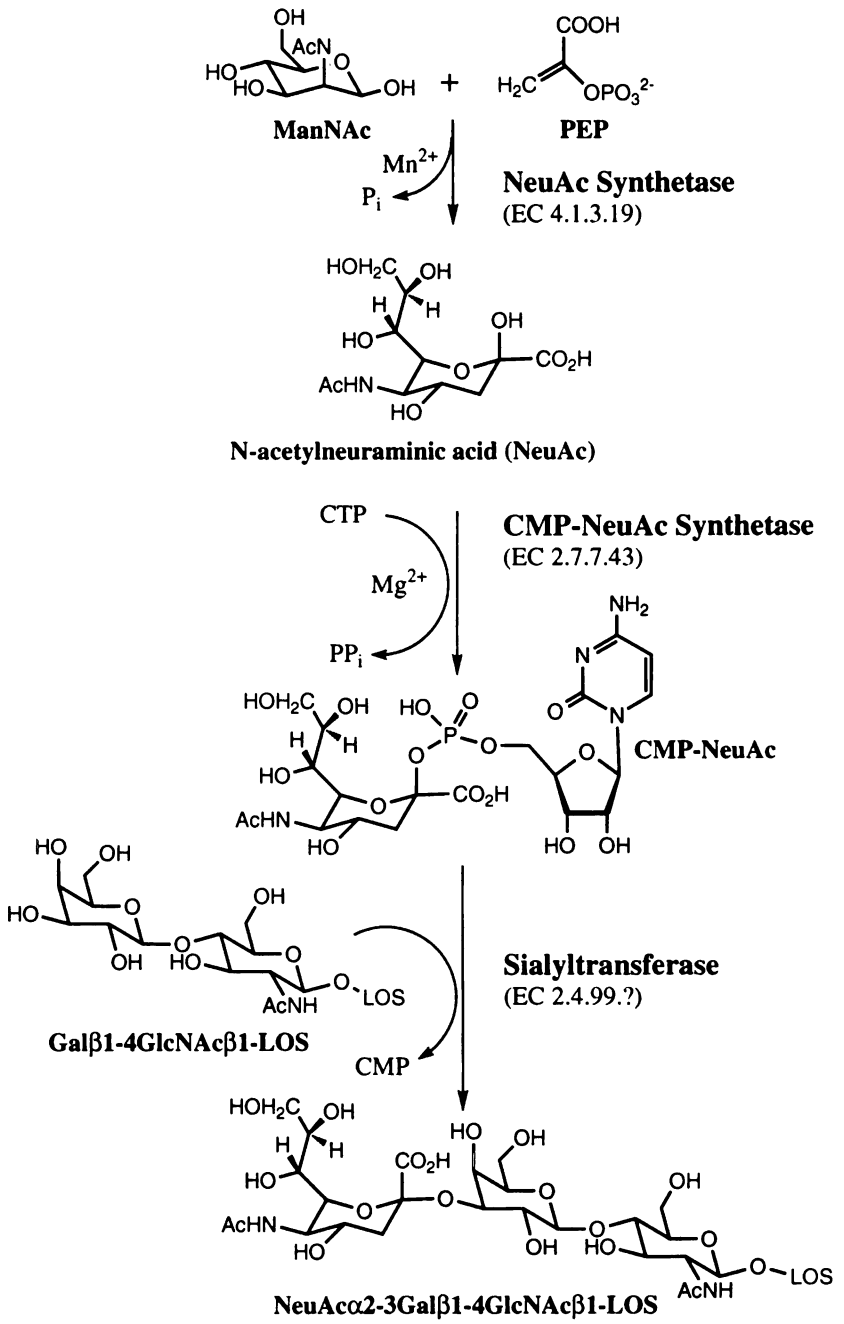


Fig. 1.4. **Sialylated LOS biosynthetic pathway.** Sialic acid containing LOS is synthesized in three steps starting from the sugar ManNAc. Only the terminal LacNAc (the acceptor for sialic acid) of the LOS molecule is shown. The enzyme committee (EC) numbers for each enzyme are shown in parenthesis. The LOS sialyltransferase is different from all previously identified animal sialyltransferases, but has not been given an EC number yet.

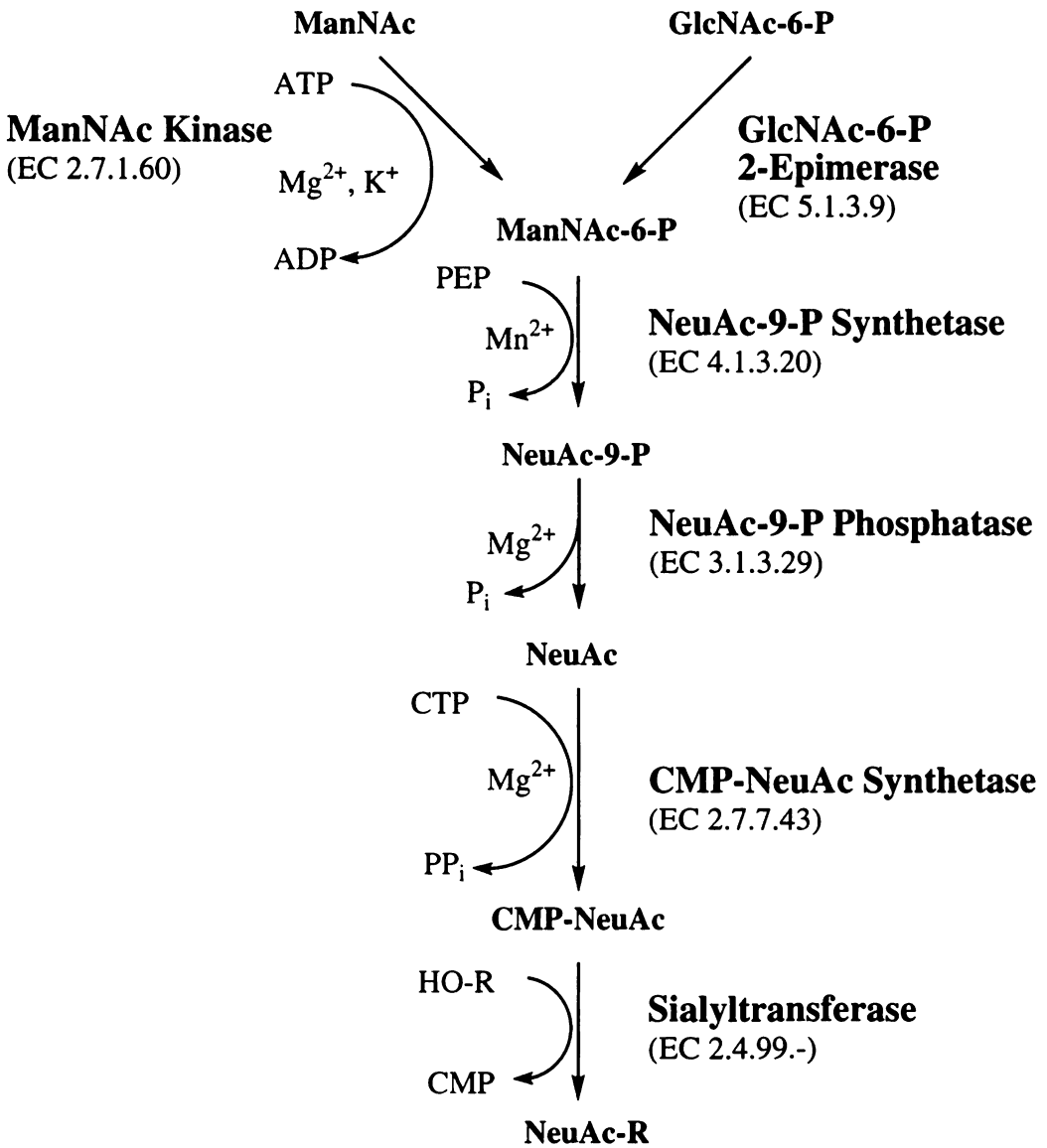


Fig. 1.5. **Biosynthetic pathway for mammalian sialylated glycoconjugates.** Mammalian glycolipids and glycoproteins (represented as R-OH, where OH is a carbohydrate hydroxyl group) are sialylated through a pathway that begins with ManNAc-6-P instead of ManNAc as for LOS. ManNAc-6-P can be synthesized from ManNAc by ManNAc kinase or by epimerization of GlcNAc-6-P. There are many different sialyltransferases (EC 2.4.99.1 - 11) that catalyze the final step that differ in their substrate specificity and the linkage that is produced (α2-3, α 2-6, or α 2-8).

were believed to code for the NeuAc synthetase based on genetic analysis, but the enzyme activity for the gene products had not been demonstrated (Vimr 1992; Ganguli et al. 1994; Vann et al. 1997). This enzyme differs from the mammalian enzyme which synthesizes NeuAc-9-P from ManNAc-6-P and PEP (Schauer 1982). NeuAc-9-P is then dephosphorylated by either a specific or non-specific phosphorylase. A specific phosphorylase has been identified for this reaction (Schauer 1982).

CMP-NeuAc synthetase catalyzes the next step in the pathway, forming the activated, nucleotide sugar used by sialyltransferases (Kean 1991). NeuAc is condensed with CTP in the presence of a divalent cation to form CMP-NeuAc and pyrophosphate. These enzymes have now been purified and characterized from several bacterial and animal sources (Vann et al. 1987; Kean 1991; Rodriguez-Aparicio et al. 1992; Ganguli et al. 1994; Haft and Wessels 1994). When this project was initiated, the only bacterial CMP-NeuAc synthetase that had been purified to homogeneity and characterized was from *E. coli* (Vann et al. 1987; Zapata et al. 1989; Ambrose et al. 1992). Although, several animal CMP-NeuAc synthetases have been characterized and partially purified, none of their genes have been cloned and only a short N-terminal sequence is available for one enzyme (Rodriguez-Aparicio et al. 1992).

The final step in the biosynthesis of sialic acid containing glycoconjugates is catalyzed by a sialyltransferase (STase) that uses CMP-NeuAc to donate NeuAc to a carbohydrate acceptor molecule. An LOS specific STase has been detected in extracts of *Neisseria* spp. and partially characterized (Mandrell et al. 1993; Mandrell et al. 1993). Just last year, the goal of many scientists for many years was attained when the meningococcal and gonococcal STase genes were cloned (Gilbert et al. 1996). The gene product does not have any homology to bacterial polysialyltransferases, the enzymes that synthesize polysialic acid capsules, or to animal STases, which is not surprising considering the fact that homology based methods had been unsuccessful in cloning the

neisserial genes. Animal STases share a region of homology which has enabled the cloning of many members of this gene family (Datta and Paulson 1995).

1.10.3 Anti-Recognition

The ability of sialic acid to mask the underlying carbohydrate epitopes to which it is attached, refereed to as anti-recognition, has been known for a long time (Schauer 1982; Schauer et al. 1984; Schauer 1985). For example, removal of sialic acid from soluble, serum glycoproteins results in their removal from circulation through receptor mediated endocytosis by hepatocytes. A galactose specific lectin, also known as the asialoglycoprotein receptor, on hepatocytes binds to the galactose residues that are exposed by removal of sialic acid (Schauer 1985). Similarly, mammalian blood cells that are desialylated are cleared from the blood stream by binding to liver, spleen, and peritoneal macrophages followed by phagocytosis. Interestingly, only 10-20% of the sialic acid needs to be removed for this to occur (Schauer 1985).

1.10.4 Recognition

Increasingly, it has been recognized that sialic acids also play a direct role in cellular recognition (Varki 1992; Varki 1993; Schauer et al. 1995; Varki 1997). The well known cholera, pertussis, and tetanus toxins all bind to sialic acid containing molecules on the cell surface (Schauer et al. 1995). Many bacteria and viruses adhere to host cells through sialic acid containing receptors, as well (Schauer et al. 1995).

1.10.5 Protection from the Host Immune Response

Sialic acid rich surfaces are capable of regulating activation of the complement system (Bitter-Suermann 1993). Polyanions on cell surfaces, particularly sialic acid, are known to bind factor H. Factor H along with factor I degrade C3b, preventing opsonization and the eventual formation of membrane attack complex on the cell surface.

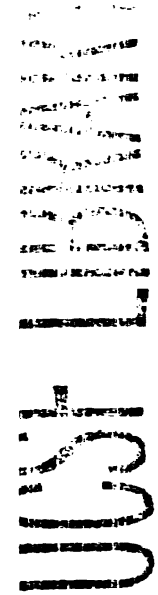
A sialic acid covered membrane is one of many ways that the host protects itself from the effects of complement activation, and this strategy has been successfully adopted by several bacterial pathogens (Bitter-Suermann 1993). The sialic acid containing capsules of *N. meningitidis*, *E. coli* K1, and group B streptococci all have been identified as important virulence factors and at least part of their function is to protect the bacteria from complement (Jennings et al. 1984; Jann and Jann 1985; Timmis et al. 1985; Bitter-Suermann 1993). Sialic acid as a component of LOS demonstrates these properties as well (Parsons et al. 1989; Elkins et al. 1992; Wetzler et al. 1992).

1.10.6 Sialic Acid as a Virulence Factor in Bacteria

Sialylation of the LOS of *N. gonorrhoeae* is now viewed as a major factor in the organism's pathogenicity (Smith et al. 1992; Rest and Mandrell 1995; Smith et al. 1995). It had been known for quite some time that *N. gonorrhoeae* isolated from patients, when tested directly, is serum resistant, but most strains become serum sensitive after subculture. In a series of studies dating back to the early 1980s, Parsons and Smith and colleagues discovered that a factor in blood and secretions could restore serum resistance to these strains, and then isolated the activity and identified it as CMP-NeuAc (Smith et al. 1992; Rest and Mandrell 1995). CMP-NeuAc is the activated nucleotide sugar that is used by sialyltransferases to donate NeuAc to a carbohydrate acceptor. Further studies have shown that only LOS is sialylated in the presence of CMP-NeuAc, that sialylated LOS is responsible for the serum resistance, and this process occurs in vivo as strains isolated from patients contain sialylated LOS (Smith et al. 1995). In contrast to *H. ducreyi*, *H. influenzae*, and *N. meningitidis*, *N. gonorrhoeae* is not capable of synthesizing CMP-NeuAc and must obtain it from the host. Many studies have examined the role of NeuAc in *N. gonorrhoeae* and have found that sialylated LOS: 1) can block the binding of bactericidal antibodies to LOS and other antigens, as well as disrupt the activation of complement (Parsons et al. 1989; Elkins et al. 1992; Wetzler et al. 1992;

Frangipane and Rest 1993; van Putten 1993; de la Paz et al. 1995) and 2) decrease opsonic and non-opsonic phagocytosis of gonococci by neutrophils (Kim et al. 1992; Rest and Frangipane 1992). The addition of sialic acid to the terminal LacNAc on the LOS does not disrupt the mimicry discussed above, as sialic acid is commonly found attached to LacNAc in the human host. In fact, sialyl-paragloboside, and longer chain variants, are the major ganglioside components of human granulocytes (Fukuda et al. 1985).

The role of sialylated LOS in *N. meningitidis* is similar to *N. gonorrhoeae*, although its effect is not as profound because of the dominant capsular virulence factor (Smith et al. 1995). All case isolates of *N. meningitidis* possess a capsule and mutants lacking capsules are avirulent in animal models demonstrating how important this component is in meningococcal disease. However, LOS, particularly sialylated LOS, still plays a significant role (Smith et al. 1995).



The role of sialic acid in *H. ducreyi* and *H. influenzae* is largely unknown. One of the goals of this project has been to better understand the role of LacNAc and sialyl-LacNAc epitopes on *H. ducreyi* LOS in the organism's pathogenesis. An interesting observation has been made about the amount of NeuAc incorporated into the LOS of *H. ducreyi* strains. In all cases, the major LOS species that contains the LacNAc acceptor is not completely converted to the sialylated version, with the highest level of incorporation being about 50% (Melaugh et al. 1996). In contrast, gonococci are capable of complete, or nearly complete, conversion of the LacNAc acceptor to sialyl-LacNAc, both in vitro and in vivo if enough CMP-NeuAc is available (Apicella et al. 1990; Mandrell et al. 1990). However, it has been suggested that low levels of NeuAc may be important in some aspects of gonococcal pathogenesis as sialylated LOS has been found to block the entry of *N. gonorrhoeae* into some human mucosal cells (van Putten 1993; van Putten et al. 1995). van Putten has proposed that *N. gonorrhoeae* may switch between an invasive state with low levels of sialylated LOS and a immuno-resistant state with high levels of

sialylated LOS (van Putten 1993). A small human challenge study has added support to this theory by showing that gonococci with sialylated LOS were less infective than unsialylated gonococci (Schneider et al. 1996). Because *H. ducreyi* produces its own CMP-NeuAc and does not seem to undergo phase variation of its LOS, it may express a heterogeneous mixture of LOS glycoforms so that its LOS can function in several roles. For example, the level of sialylation may be controlled so that it offers the bacterium protection from host defenses while not interfering with the adherence or infectivity function of unsialylated LOS or outer membrane components. It should be emphasized that small changes in the level of sialic acid on erythrocyte membranes (a decrease of 10-20%) results in a rapid biological effect (their clearance from the blood stream) (Schauer 1985). A recent study has shown that a *H. ducreyi* isogenic mutant that cannot synthesize the terminal LacNAc component, and therefore the sialyl-LacNAc component as well, of its LOS shows much decreased adherence to and invasion of keratinocytes (Gibson et al. 1997). The isogenic *H. ducreyi* mutant with a defective CMP-NeuAc synthetase that we have created in the course of this project is being tested in adherence and infectivity models, for its resistance to serum and neutrophil killing, and in human and animal models of chancroid. These studies will now allow us to determine the role of sialylated LOS in *H. ducreyi* pathogenesis.

CHAPTER 2.

Purification and Characterization of a Cytidine 5'-Monophosphate N-Acetylneuraminic Acid Synthetase from *Haemophilus ducreyi*

2.1 INTRODUCTION

As a first step in elucidating the role of sialic acid in the biology of *H. ducreyi*, we attempted to identify and isolate a central enzyme involved in the biosynthesis of sialic acid containing glycoconjugates. That enzyme, N-acetylneuraminic acid cytidylyltransferase (EC 2.7.7.43) (CMP-NeuAc synthetase), catalyzes the reaction of CTP and NeuAc in the presence of divalent cations to form cytidine monophosphate N-acetylneuraminic acid (CMP-NeuAc), which is the nucleotide sugar donor used by sialyltransferases in the final step of the biosynthesis of sialylated glycoconjugates. The initial stimulus for this project was the recent discovery made in our laboratory, and in collaboration with others, demonstrating that the outer membrane lipooligosaccharides (LOS) of *H. ducreyi* contained terminal NeuAc attached to N-acetyllactosamine forming epitopes that are structurally and immunochemically similar to human antigens. Although the role of sialic acid in the pathogenesis of *Neisseria gonorrhoeae* (an organism that produces LOS structurally similar to *H. ducreyi*) was beginning to be elucidated, nothing was known about its role in *H. ducreyi* pathogenesis at this time. Furthermore, *H. ducreyi* was known to produce sialylated LOS endogenously, while *N. gonorrhoeae* requires CMP-NeuAc from the host, making comparison of the two systems of some interest. Along with this, the numerous examples of the importance of sialic acid in many other instances made work on determining its role in *H. ducreyi* compelling.

Isolation and sequencing of an enzyme in the sialic acid biosynthetic pathway, such as the CMP-NeuAc synthetase, would allow for the cloning of its gene and the

creation of an isogenic mutant unable to add sialic acid to its LOS. This mutant would permit a thorough study of the biological roles of sialic acid, and the underlying N-acetyllactosamine to which it is normally attached, in the LOS of *H. ducreyi.* Furthermore, because only two bacterial CMP-NeuAc synthetases had been characterized at this time, and only one purified to homogeneity, characterization and sequencing of another bacterial enzyme would enhance the general understanding of this enzyme class and be helpful in finding highly conserved residues and regions of these enzymes. This chapter details the purification of a CMP-NeuAc synthetase from a *H. ducreyi* strain 35000 cell lysate, its characterization, and N-terminal sequencing.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Cibacron Blue 3GA-Agarose, Reactive Green 19-Agarose, Reactive Yellow 86-Agarose, Reactive Red 120-Agarose, Reactive Brown 10-Agarose, Reactive Blue 4-Agarose, CTP, N-acetylneuraminic acid, N-glycolylneuraminic acid, 2-keto-3-deoxy-D-manno-octulosonic acid, and tetrabutylammonium bromide were from Sigma. *H. ducreyi* strain 35000 was originally isolated in Winnipeg, Canada in 1975 and was obtained from Dr. Anthony Campagnari (SUNY at Buffalo).

2.2.2 Methods

2.2.2.1 *Growth of Bacteria*

H. ducreyi strain 35000 was grown on chocolate agar plates (GC Medium Base (Difco), 1% (w/v) hemoglobin (Difco), and 1% (v/v) IsoVitaleX (Becton Dickinson) or 0.1% (w/v) glucose, 0.01% (w/v) L-glutamine, 0.026% (w/v) L-cysteine hydrochloride)

incubated in candle jars at 33-35°C for 1-2 days. The bacteria were suspended in 50 mM Bis-Tris-HCl, pH 6.5 (buffer A) and used immediately for purification of the CMP-NeuAc synthetase or frozen and stored at -80°C.

2.2.2.2 *Purification of CMP-NeuAc Synthetase from H. ducreyi Cell Lysate*

The bacterial suspension (3 g wet weight cells in 20 ml buffer A) was adjusted to 2 mM EDTA and 0.2 mg/ml lysozyme and was stirred for 20 min at room temperature. Unlysed cells and cellular debris were removed by centrifugation at 13,000 x g for 10 min. The supernatant was applied to two 5 ml EconoPac Q cartridges (BioRad) connected in series and equilibrated in buffer A. The enzyme was eluted with 0.2 M NaCl in buffer A. The active fraction (11 ml) was applied to a 5 ml Cibacron Blue 3GA-Agarose dye column also equilibrated with buffer A. The column breakthrough (20 ml), which contained most of the enzyme activity, was concentrated to 0.5 ml with two Centricon-30 concentrators (Amicon) and applied to two Bio-Sil TSK-125 (BioRad; 7.5 mm x 600 mm each) size exclusion columns connected in series and equilibrated with 20 mM KH_2PO_4, 0.5 mM EDTA, 20% (v/v) glycerol, pH 7.0. The active fractions (4.5 ml) were pooled, 0.5 ml of 0.5 M Tris-HCl, pH 8.5 was added to increase the pH, and the sample applied to a MonoQ HR 5/5 strong anion exchange (trimethyl ammonium) column (Pharmacia; 5 mm x 50 mm) equilibrated with 50 mM Tris-HCl, 20% (v/v) glycerol, pH 8.0. The enzyme was eluted with a KCl gradient (0.5 ml/min, 50-200 mM KCl in 40 min) in the same buffer. The active fractions (3 ml) were pooled, concentrated to 0.4 ml with a Centricon-30 concentrator, and adjusted to 1.5 M $(NH_4)_2SO_4$. The sample was applied to a Hydropore-5-HIC (hydrophobic interaction chromatography, polyethylene glycol bonded resin) column (Rainin; 4.6 mm x 100 mm) equilibrated with 0.1 M KH_2PO_4, 0.5 mM EDTA, 1.2 M $(NH_4)_2SO_4$, pH 7.0 and eluted with a gradient of 0.1 M KH_2PO_4, 0.5 mM EDTA, pH 7.0 (0.5 ml/min, 1.2 - 0 M $(NH_4)_2SO_4$ in 40 min). The active fractions (3 ml) were readjusted to 1.2 M $(NH_4)_2SO_4$ and rechromatographed on the hydrophobic interaction chromatography (HIC) column using a shallower gradient

(0.4 ml/min, 1.125 - 0.6 M $(NH_4)_2SO_4$ in 52.5 min). The active fractions (2.4 ml) were pooled, concentrated and desalted with a Centricon-30 concentrator to a volume of 0.5 ml, and applied to a C4 reverse phase HPLC column (Vydac; 2.1 mm x 150 mm). The C4 column was equilibrated with 0.1% trifluoroacetic acid (TFA) and the proteins were eluted with a gradient of 0.08% TFA, 70% CH_3CN over 70 min at a flow rate of 0.2 ml/min.

2.2.2.3 *Amino Acid Sequencing*

The three major peaks eluted from the HPLC C4 column were each subjected to N-terminal sequence analysis using an Applied Biosystems 470A gas-phase sequencer with an on-line ABI Model 130A PTH analyzer. For the fraction identified as containing the CMP-NeuAc synthetase, forty-three cycles were obtained in which the amino acid residue could be assigned with confidence. The first cycle produced 4 pmol of methionine with the yield dropping to 0.1 pmol phenylalanine by the 43rd cycle. Based on typical conversion yields (30-40%), approx. 10-13 pmol (0.25-0.33 μg) of CMP-NeuAc synthetase was loaded on the sequencer.

2.2.2.4 *Affinity of CMP-NeuAc Synthetase for Dye Columns*

In order to identify suitable dye columns for use in the purification of the CMP-NeuAc synthetase a small trial with six different resins was conducted (see Table 2.1 for dye resins used). Supernatant from a *H. ducreyi* cell lysate (250 μg of protein in 50 mM Tris-HCl pH 8.0, 5 mM dithiothreitol) was loaded on to 0.5 ml agarose columns with covalently bound dyes equilibrated with 50 mM Tris-HCl pH 8.0, 1 mM dithiothreitol. Unbound protein was washed through the column with 2 ml of the equilibration buffer. Protein bound to the dye column was eluted with 2 ml of the same buffer containing 1 M NaCl. The bound and unbound fractions were assayed for protein content and CMP-NeuAc synthetase activity.

2.2.2.5 *CMP-NeuAc Synthetase Assay*

The CMP-NeuAc synthetase activity of lysates and column fractions was assayed by the following procedure: 4 µl of enzyme was mixed with 6 µl of assay mix and incubated at 37°C for 10-120 min. The reaction was stopped by diluting to 500 µl with cold water and placing on ice. If not immediately analyzed, the samples were frozen for later analysis. The final concentrations of components in the reaction were 1 mM CTP, 2 mM NeuAc, 20 mM $MgCl_2$, 0.2 M Tris-HCl pH 8.0 (at 37°C). The amount of CMP-NeuAc product formed in the reaction was determined by one of the two following HPLC methods. For both methods, frozen assay solutions were thawed directly before analysis and a 20 µl loop was completely filled by a 100 µl injection on a Rainin (Woburn, MA) HPLC system.

2.2.2.6 *Reverse Phase HPLC Method*

The first method used was a modification of a method developed by Petrie and Korytnyk in which CMP-NeuAc is separated from CTP and other cytidine nucleotides on a reverse phase column using ion pair chromatography (Petrie and Korytnyk 1983). A C18 reverse phase column (Vydac, 2.1 x 50 mm) was operated at a flow rate of 0.5 ml/min with detection at 270 nm. Solvent A was 10 mM tetrabutylammonium bromide, 10 mM KH_2PO_4 pH 7.0 in H_2O and solvent B was 10 mM tetrabutylammonium bromide, 10 mM KH_2PO_4 pH 7.0 in 50% CH_3CN. The gradient separation was completed over 6.5 min.

2.2.2.7 *Anion Exchange HPLC Method*

An improved HPLC method used a nucleotide analysis column (low capacity anion exchange silica based resin) (Vydac; 4.6 x 50 mm) to resolve CMP-NeuAc from the substrate CTP and any small amounts of CMP or CDP present. Solvent A was water and solvent B was 0.5 M ammonium formate (NH_4HCO_2) (adjusted to pH 3.5 with formic acid). The column was operated at a flow rate of 2 ml/min and the separation was

complete in 2 min followed by a 1.5 min reequilibration cycle. Quantitation of the CMP-NeuAc peak area was done using a standard curve of CMP (Petrie and Korytnyk 1983). Detection was at 280 nm (λ_{max} of CMP and CMP-NeuAc at pH 3.5) using an Applied Biosystems 1000S diode array detector and was linear from 5-10,000 pmol. A unit is defined as the amount of enzyme that catalyzes the formation of one μmol of CMP-NeuAc per min under the conditions of the assay.

2.2.2.8 *pH Optimum*

To determine the pH optimum of the CMP-NeuAc synthetase, enzyme activity was assayed in a similar manner as described above, but using a three component buffer system that produces a constant ionic strength over a wide range of pH values (Ellis and Morrison 1982). Briefly, 2 μl of enzyme was mixed with 18 μl of assay mix, incubated at 37°C for 30 min, and stopped by diluting to 100 μl with cold water and placing on ice. The final concentrations of components in the reaction were 1 mM CTP, 5 mM NeuAc, 20 mM $MgCl_2$, 100 mM ACES, 52 mM Tris, and 52 mM ethanolamine. The pH at 37°C ranged from 6.24 to 9.78. The assays were analyzed by the anion exchange HPLC method.

2.2.2.9 *Divalent Cation Requirement*

CMP-NeuAc synthetase was incubated at 37°C for 30 min with 0-20 mM $MgCl_2$, $MnCl_2$, or $CaCl_2$. The final concentration of other components in the reaction were 1 mM CTP, 5 mM NeuAc, and 0.2 M MOPS pH 7.2 (at 37°C). The assays were analyzed by the anion exchange HPLC method.

2.2.2.10 *Effect of Ionic Strength on CMP-NeuAc Synthetase Activity*

In order to determine the effect of ionic strength on the activity of the CMP-NeuAc synthetase, the enzyme (5 μl) was incubated with 0-0.8 M KCl or $(NH_4)_2SO_4$ included in the reaction with the other components in a final volume of 50 μl. The final

concentration of components in the reaction were 10 mM CTP, 10 mM NeuAc, 20 mM $MgCl_2$, and 0.2 M MOPS pH 7.2 (at 37°C). The reaction was stopped by diluting 10 or 20 µl to 1000 µl with cold water and placing on ice. The assays were analyzed by the reverse phase HPLC method.

2.2.2.11 *Protein Determination*

Protein was determined using the BioRad DC Protein Assay (Alam 1992) according to the manufacturer's instructions. BSA was used as a standard.

2.3 RESULTS AND DISCUSSION

CMP-NeuAc Synthetase Assay. Usually, CMP-NeuAc synthetase activity is determined by the periodic acid/thiobarbituric acid colorimetric assay developed by Warren (reviewed in (Reuter and Schauer 1994)). The micromethod requires 100 µl of sample and has a detection limit of 16 µM. The procedure requires several mixing and heating steps along with an extraction into organic solvent before measuring absorbance. Radiometric assays requiring separation of components by paper or thin layer chromatography and HPLC assays have also been described, but less frequently used (Petrie and Korytnyk 1983; Rodriguez-Aparicio et al. 1992; Haft and Wessels 1994).

To find improved mthods for assaying CMP-NeuAc synthetase activity, I developed two separate HPLC methods. The first was a modification of a published method that used ion pair chromatography with a reverse phase column (Petrie and Korytnyk 1983). Under the modified conditions, gradient elution of all components was complete in 6.5 min (Fig. 2.1A) as compared to a 20 min gradient in the original work. However, use of a reverse phase column was unsatisfactory, especially with crude samples, because proteins and lipids will readily bind to the column. Very harsh conditions are often necessary to remove these contaminants from the column or else

performance will deteriorate. A method for cleaning up the samples before injection could have been developed, but it would have only added to the time requirements of the assay. Furthermore, a 6.5 min gradient followed by reequilibration of the column was still a fairly long time when many enzyme assays needed to be conducted. Therefore, an anion exchange HPLC method was developed that used a Vydac nucleotide analysis column and gradient elution of all components in 2 min (Fig. 2.1B). Column fouling was avoided because very few proteins will bind to anion exchange resins at pH 3.5, the pH of the ammonium formate buffer. A previously described anion exchange HPLC method (using a different HPLC column) for assaying CMP-NeuAc synthetase required a 15 min gradient with no mention of reequilibration time (Rodriguez-Aparicio et al. 1992).

The amount of CMP-NeuAc produced in reactions was quantitated with a standard curve of CMP, assuming equal extinction coefficients for CMP and CMP-NeuAc (Petrie and Korytnyk 1983). For the anion exchange method, the detector response was linear from 5 to 10,000 pmol of CMP loaded on the column (Fig. 2.2). For most assays, a 20 µl loop was completely filled with a 100 µl injection. A detection limit of 5 pmol of CMP-NeuAc in 20 µl, equates to 0.25 µM which is 64 times more sensitive than the periodic acid/thiobarbituric acid micromethod mentioned above. For even greater sensitivity, a 50 µl loop was sometimes used that lowered the detection limit to 0.1 µM, 160 times more sensitive than the colorimetric assay. This increased sensitivity allowed for very small amounts of enzyme and assay components to be consumed in individual assays. Typically, only 4 µl of enzyme was used in a final volume of 10 µl when following enzyme activity in chromatography fractions.

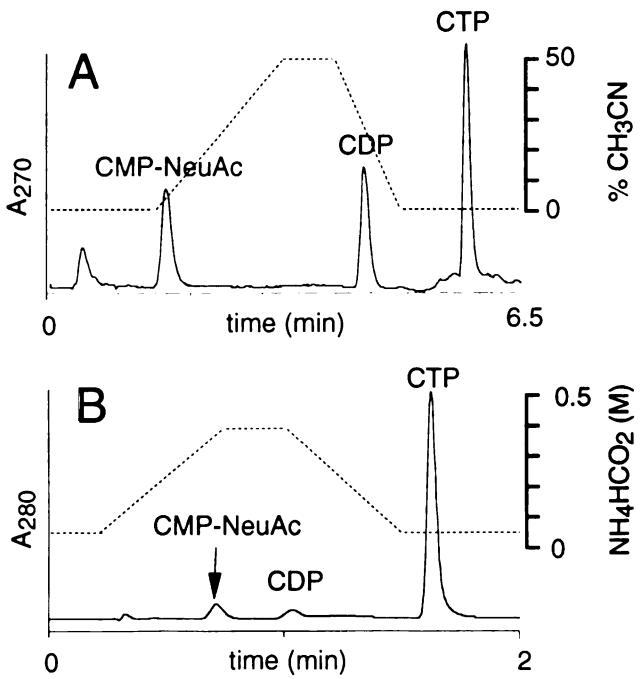


Fig. 2.1. **HPLC chromatograms of enzyme assays based on ion pair and anion exchange chromatography.** (A) The product, CMP-NeuAc, was separated from CDP and CTP in 6.5 min using a Vydac C18 column (2.1 x 50 mm), 10 mM tetrabutylammonium bromide as the ion pair reagent, and a gradient of CH_3CN. (B) CMP-NeuAc, was separated from CDP and CTP in under 2 min with a Vydac nucleotide analysis column (4.6 x 50 mm) using a gradient of NH_4HCO_2/HCO_2H. CMP (none present), elutes before CMP-NeuAc and is also baseline resolved in both methods.

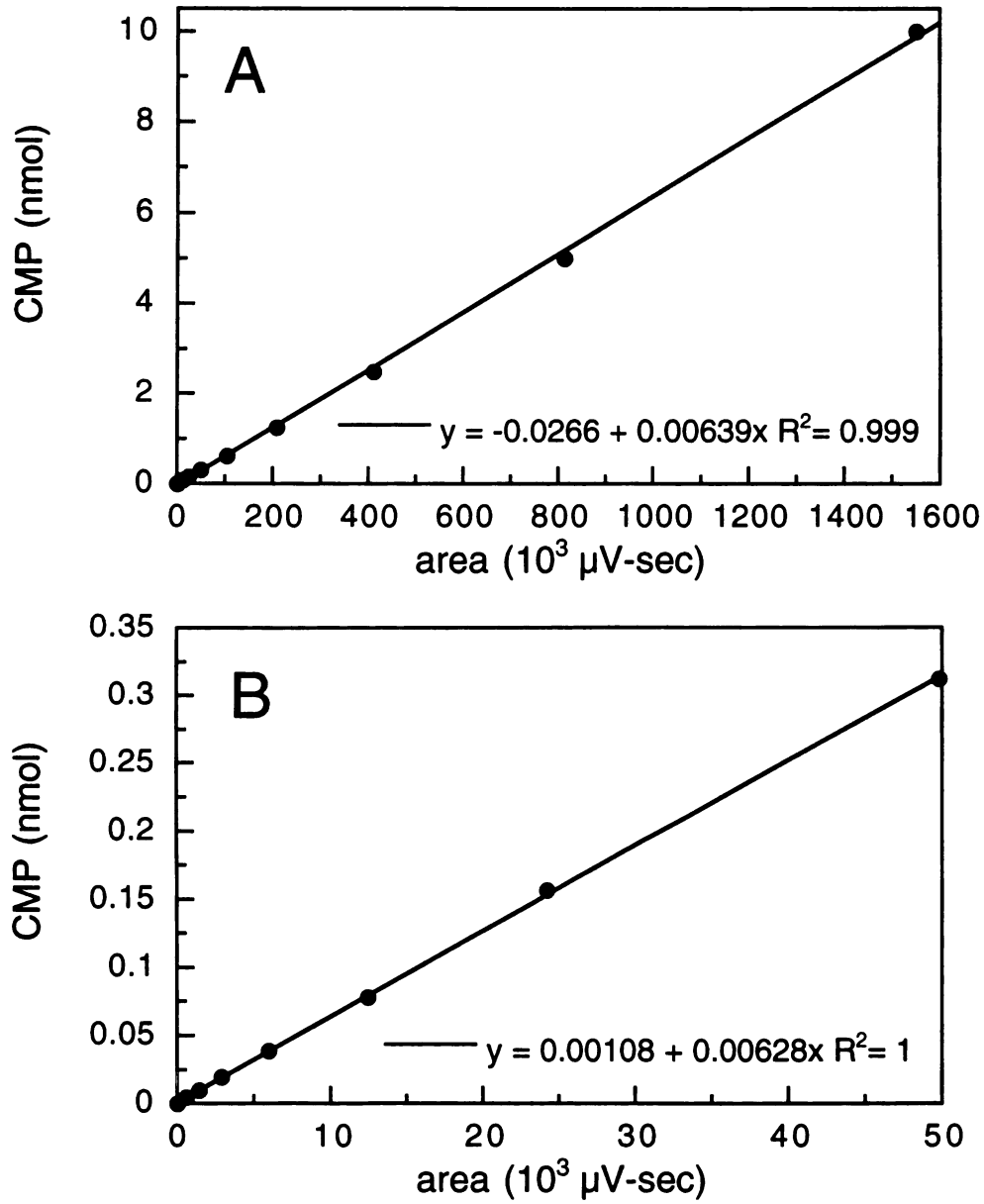


Fig. 2.2. **Standard curve of CMP with the Vydac nucleotide analysis column and ABI 1000S diode array detector.** Serial dilutions of CMP were injected on the anion exchange column (Vydac; 4.6 x 50 mm) by filling a 20 µl loop with a 100 µl injection. (A) The detector response of the ABI 1000S diode array detector at 280 nm was linear from 0.005 to 10 nmol of CMP. (B) Plot of the same data expanding the region of low CMP concentration (0.005-0.31 nmol). Only the data points shown were used in the least squares fit of the line.

Purification of the CMP-NeuAc Synthetase. A CMP-NeuAc synthetase from the *H. ducreyi* cell lysate was purified to homogeneity using a procedure consisting of anion exchange, dye, size exclusion, hydrophobic interaction, and reverse phase chromatography (Fig. 2.3). Seven chromatographic steps were used to obtain the purified product. Enzyme activity was followed throughout the purification with the exception of the final step of reverse phase chromatography which required the use of denaturing conditions (low pH and organic solvent). This final step resolved three major peaks which were sequenced by Edman degradation. The CMP-NeuAc synthetase was identified by homology to the two other known CMP-NeuAc synthetases from *Escherichia coli* K1 and *Neisseria meningitidis* (Fig. 2.4). Unfortunately, because of very low amounts of protein in the final steps and no activity after reverse phase chromatography a quantitative analysis of the purification fold and yield could not be completed. It was estimated form the Edman sequencing that less than 0.5 μg of enzyme was applied to the sequencer. Furthermore, silver staining was required for visualization of a 5% aliquot of the sample that was analyzed by SDS-PAGE. The staining revealed two bands very close together, the more intense one at 27.0 kDa and a fainter one at 26.1 kDa (data not shown). Only one sequence was detected during Edman degradation so the smaller molecular weight band may have been a slightly degraded form of the enzyme. A molecular weight of 27 kDa was very similar to that of the *N. meningitidis* enzyme (25 kDa) (Ganguli et al. 1994). The *E. coli* enzyme has a molecular weight of 49 kDa (Zapata et al. 1989).

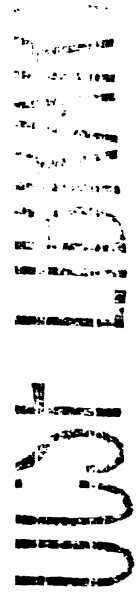
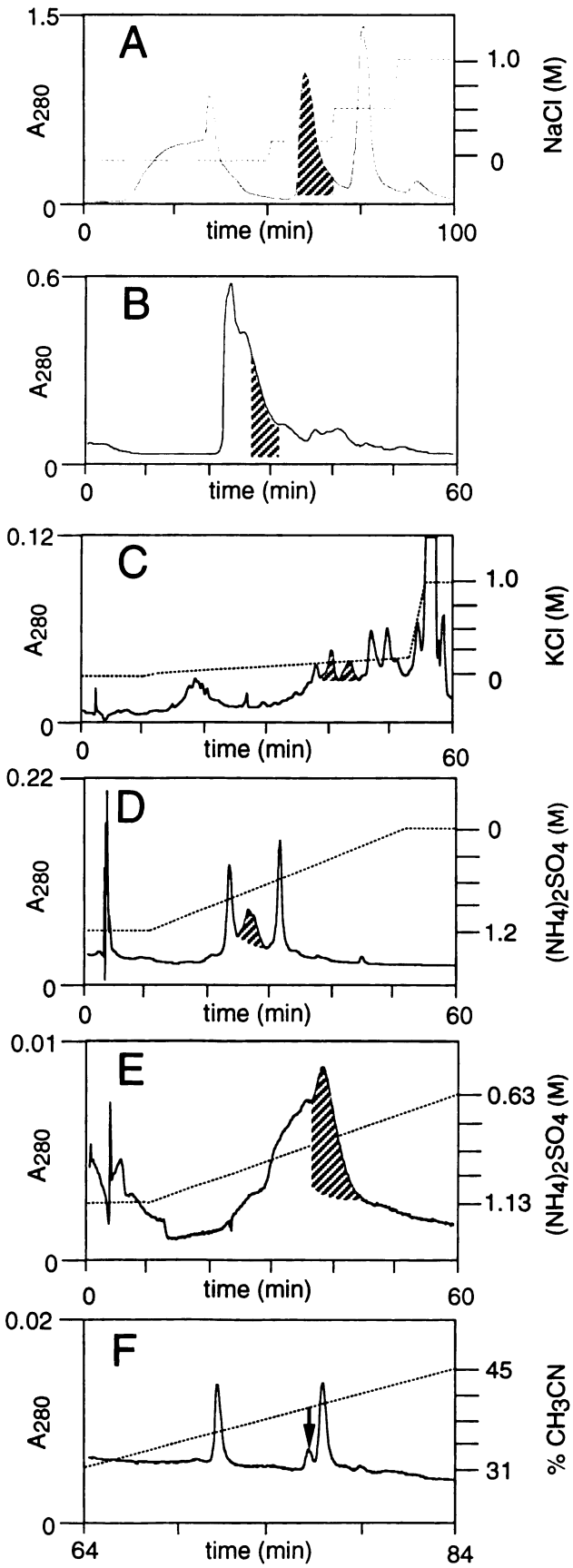


Fig. 2.3. **Purification of the CMP-NeuAc synthetase from a *H. ducreyi* cell lysate.** The fractions containing enzyme activity are shaded. See section 2.2.2.2 in "Methods" for experimental details. (A) Anion exchange chromatography of the *H. ducreyi* lysate on two 5 ml EconoPac Q columns connected in series. (B) Active fractions from the initial anion exchange column were passed through a Blue dye column and then subjected to size exclusion chromatography. (C) Anion exchange chromatography using a MonoQ HR 5/5 column of active fractions from the size exclusion column. (D) Hydrophobic interaction chromatography of active fractions from the MonoQ column. (E) Rechromatography of active fractions from (D) on the HIC column using a shallower gradient. (F) The fractions containing enzyme activity from (E) were pooled, desalted, and loaded on a Vydac C4 reverse phase column. After Edman sequencing, the CMP-NeuAc synthetase was identified as peak 2 (indicated by arrow) by its homology to other CMP-NeuAc synthetases (see Fig. 2.4).


A
1.5
0
A280
0
time (min)
100
1.0
0
NaCl (M)
B
0.6
0
A280
0
time (min)
60
C
0.12
0
A280
0
time (min)
60
1.0
0
KCl (M)
D
0.22
0
A280
0
time (min)
60
0
1.2
(NH4)2SO4 (M)
E
0.01
0
A280
0
time (min)
60
0.63
1.13
(NH4)2SO4 (M)
F
0.02
0
A280
64
time (min)
84
45
31
% CH3CN

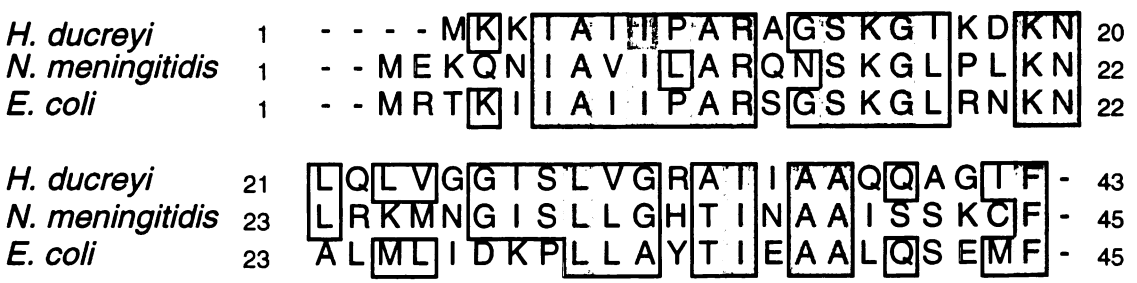


Fig. 2.4. **Sequence alignment of the N-terminal sequence data with CMP-NeuAc synthetases from *E. coli* K1 and *N. meningitidis*.** The *H. ducreyi* protein isolated in the final step of reverse phase chromatography (Fig. 2.3F) was identified as a CMP-NeuAc synthetase by its homology to the sequences from the two known bacterial CMP-NeuAc synthetases. Residues identical in all three sequences are shaded and boxes enclose regions of similarity.

A review of the purification protocols used for CMP-NeuAc synthetases from bacterial and animal sources revealed that anion exchange and dye chromatography were often useful purification steps (Warren and Blacklow 1962; Kean and Roseman 1966; Vann et al. 1987; Shames et al. 1991; Liu et al. 1992; Rodriguez-Aparicio et al. 1992; Schmelter et al. 1993). The *H. ducreyi* enzyme was no exception and both types of chromatography were used effectively in purification trials. The enzyme bound to a strong anion exchanger even at the somewhat low pH of 6.5 (Fig. 2.3A). The high resolution MonoQ anion exchanger was particularly useful in removing large amounts of contaminating proteins (Fig. 2.3C).

Although not used to great effect in this purification scheme, dye columns were used successfully at other times in the purification of the native enzyme and, as will be discussed in the next chapter, a Reactive Green 19 dye column was a key step in the purification of the recombinant enzyme. In a screening of several dye columns for enzyme binding, it was found that most (4 of 6) bound the enzyme completely at pH 8.0, one bound the enzyme partially, and one not at all (Table 2.1). The Reactive Yellow 86 dye column that did not bind the enzyme at all, also bound very little other protein so probably would not have been useful for removing contaminants. The two best dye columns, as judged by their ability to retain enzyme activity while binding the least amount of total protein, were the Cibacron Blue 3GA and Reactive Green 19 columns. In the scheme presented here, the CMP-NeuAc synthetase did not bind to the Cibacron Blue 3GA column, but the enzyme was applied in pH 6.5 buffer with 0.2 M NaCl, conditions that had not been tested up to that time.

Table 2.1. Affinity of CMP-NeuAc synthetase for several dye columns

Dye Columns	% of protein in unbound fraction	activity in unbound fraction [a]	activity in bound fraction
Cibacron Blue 3GA-Agarose	92	-	+
Reactive Green 19-Agarose	84	-	+
Reactive Blue 4-Agarose	80	-	+
Reactive Brown 10-Agarose	52	-	+
Reactive Red 120-Agarose	60	+	+
Reactive Yellow 86-Agarose	84	+	-

[a] (-) No CMP-NeuAc synthetase activity detected. (+) CMP-NeuAc synthetase activity detected.

CMP-NeuAc Synthetase Properties. The relatively crude preparation of enzyme eluted from the first anion exchange column was stable to storage as a 65% ammonium sulfate suspension or 20% glycerol solution at 4°C or as a 50% glycerol solution at -20°C and -80°C for at least several months. This material was used for initial characterization of the enzyme's properties. The activity of the CMP-NeuAc synthetase was linear with time and with enzyme concentration (data not shown). The enzyme has a rather broad pH optimum from 8 to 9.5 which is similar to other CMP-NeuAc synthetases (Fig 2.5) (Kean 1991; Haft and Wessels 1994). A three component buffer system that maintains a constant ionic strength over a wide range of pH values (Ellis and Morrison 1982) was used in these experiments as the CMP-NeuAc synthetase activity was found to be inhibited by increasing ionic strength. The enzyme activity as a function of KCl and $(NH_4)_2SO_4$ concentration and ionic strength is shown in Fig. 2.6. Although, the pH of the MOPS buffer used in these experiments would have been affected by the changing ionic strength, the highest increase would have been only 0.16 pH units (see (Stevens 1992) for the effect of ionic strength on buffers). Any effect would have been to increase enzyme activity, but activity decreased steadily with ionic strength indicating that small pH changes had a negligible role. The CMP-NeuAc synthetase was fairly stable to heat denaturation. When heated at 60°C for 10 min, the enzyme retained greater than 80% of the non-heated control (data not shown). Also, the enzyme reaction at 37°C was found to be linear for at least 10 hrs under conditions generating less than 25% product. The enzyme has a requirement for Mg^{2+}, with 20 mM giving the most activity under the assay conditions used. Mn^{2+} is only 40% as effective at 20 mM, while Ca^{2+} gave no reaction (Fig. 2.7). The divalent cation requirements of other bacterial CMP-NeuAc synthetases are similar, while enzymes from animal sources are capable of using Ca^{2+} and other divalent cations in addition to Mg^{2+} and Mn^{2+} (Rodriguez-Aparicio et al. 1992; Haft and Wessels 1994). Sulfhydryl reagents are necessary for or greatly stimulate the enzyme activity of some other CMP-NeuAc synthetases (Kean 1991; Haft and Wessels 1994).

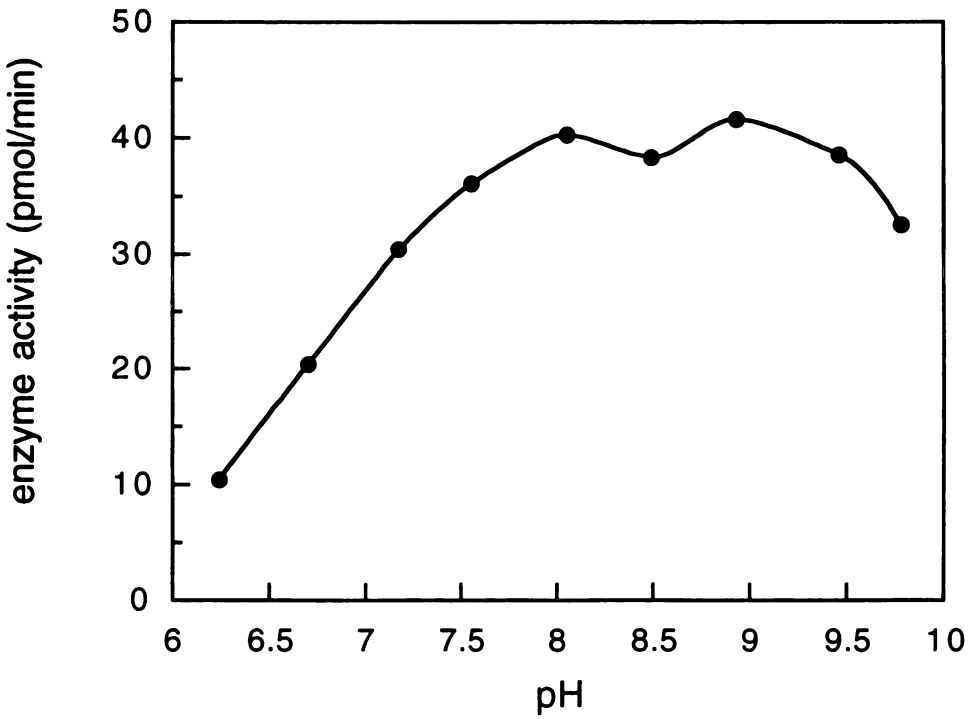


Fig. 2.5. **Effect of pH on CMP-NeuAc synthetase activity.** The CMP-NeuAc synthetase was assayed using 1 mM CTP, 5 mM NeuAc, 20 mM $MgCl_2$ in a volume of 20 µl for 30 min at 37°C. A buffer system (100 mM ACES/52 mM Tris/ 52 mM ethanolamine) with a constant ionic strength over the pH range tested was used in these experiments. A pH maxima from about pH 8 to 9.5 was observed.

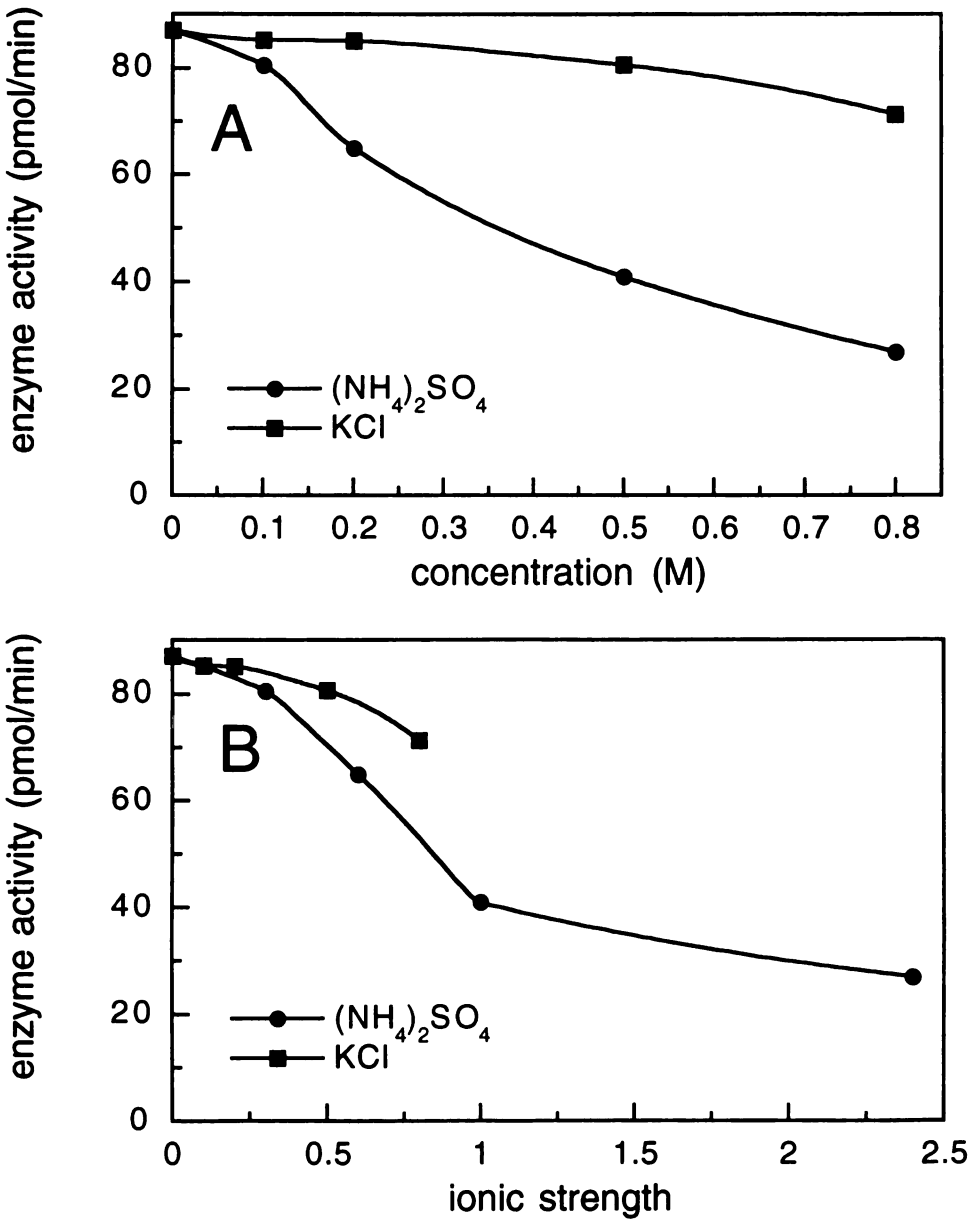


Fig. 2.6. **Effect of ionic strength on CMP-NeuAc synthetase activity.** CMP-NeuAc synthetase was assayed in the presence of KCl or $(NH_4)_2SO_4$ along with 10 mM CTP, 10 mM NeuAc, 20 mM $MgCl_2$, and 0.2 M MOPS, pH 7.2 (at 37°C) for 300 min at 37°C. (A) Plot showing the decrease in activity due to increasing concentrations of KCl and $(NH_4)_2SO_4$. (B) Same data as in (A) plotted as a function of ionic strength ($I = 0.5 \sum (c_i z_i^2)$; where c_i is the concentration and z_i is the charge of the ion).

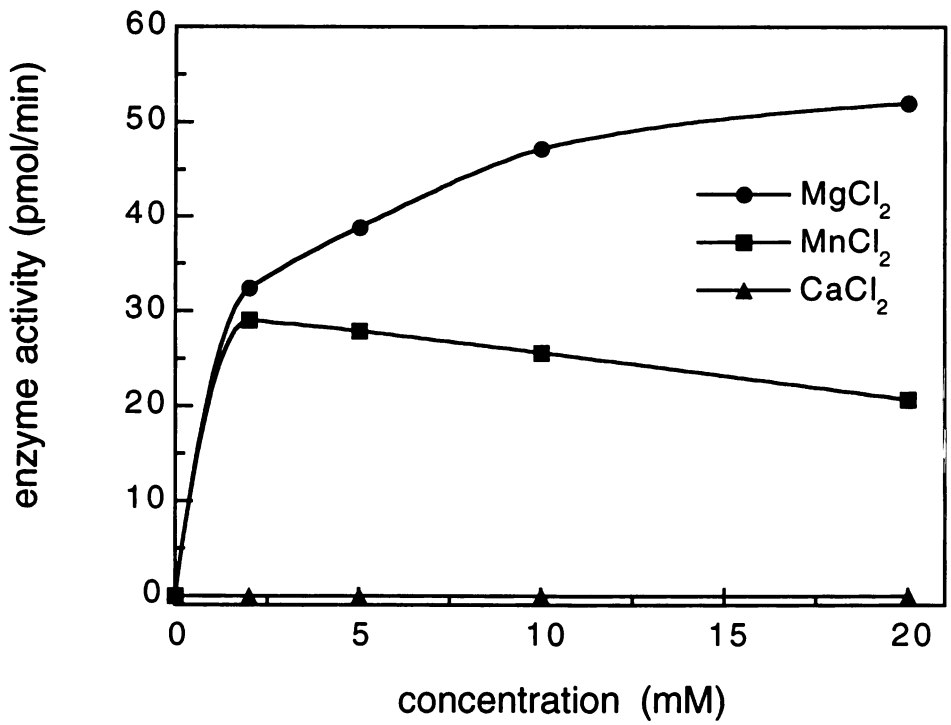


Fig. 2.7. **Effect of divalent cations on CMP-NeuAc synthetase activity**. CMP-NeuAc synthetase was assayed at 37°C for 30 min with 0-20 mM $MgCl_2$, $MnCl_2$, or $CaCl_2$ and 1 mM CTP, 5 mM NeuAc, and 0.2 M MOPS, pH 7.2 (at 37°C). A plot of enzyme activity as a function of divalent cation concentration shows, as for other CMP-NeuAc synthetases, the *H. ducreyi* CMP-NeuAc synthetase required a divalent cation for activity. The enzyme could use $MgCl_2$ and $MnCl_2$, but not $CaCl_2$. No other cations were tested.

However, dithiothreitol was found to have little effect on enzyme activity and so was not included in purification buffers or enzyme assays after initial experiments. Interestingly, N-glycolylneuraminic acid (NeuGc) was also a substrate for the *H. ducreyi* CMP-NeuAc synthetase (data not shown). NeuGc can be used as a substrate by CMP-NeuAc synthetases from animal tissues, but not by the enzymes from *E. coli* or *N. meningitidis* (Kean 1991). The monosaccharide, 2-keto-3-deoxy-D-manno-octulosonic acid (KDO), was not a substrate.

In conclusion, CMP-NeuAc synthetase activity was identified in *H. ducreyi* cell lysates, the enzyme purified to homogeneity, and 43 residues of N-terminal sequence obtained (almost 20% of the complete sequence expected based on a molecular weight of 27 kDa). The molecular weight was much smaller than the *E. coli* enzyme, but very similar to the *N. meningitidis* enzyme. The enzyme has similar characteristics to these other bacterial enzymes, with the notable exception of its ability to utilize NeuGc as a substrate. As will be seen in the next chapter, the extensive N-terminal sequence data allowed for the cloning of the CMP-NeuAc synthetase gene from a *H. ducreyi* genomic DNA library. Also, the rapid anion exchange HPLC assay developed and the knowledge gained about the native enzyme's chromatographic and enzymatic properties allowed for the rapid purification to homogeneity of the recombinant CMP-NeuAc synthetase and its further characterization.

CHAPTER 3.

Cloning and Expression of a Cytidine 5'-Monophosphate N-Acetylneuraminic acid synthetase from *Haemophilus ducreyi*: Purification to Homogeneity and Further Characterization

3.1 INTRODUCTION

The purification and characterization of a CMP-NeuAc synthetase from *H. ducreyi* was described in the previous chapter. The substantial N-terminal sequence data obtained on the native enzyme allowed us to continue our investigation into the role of sialic acid in *H. ducreyi* pathogenesis by cloning the CMP-NeuAc synthetase gene. Degenerate oligonucleotide probes derived from the N-terminal sequence data were used to successfully clone the gene from a *H. ducreyi* genomic DNA library. The nucleotide sequence of the gene was determined and the derived amino acid sequence of the CMP-NeuAc synthetase gene was found to have homology to other CMP-NeuAc synthetases and to a lesser extent to CMP-2-keto-3-deoxy-D-manno-octulosonic acid (CMP-KDO) synthetases.

To further characterize the *H. ducreyi* CMP-NeuAc synthetase, the gene was cloned into a T7 expression vector, the protein expressed in *Escherichia coli*, and purified to apparent homogeneity by anion exchange, Green 19 dye, and hydrophobic interaction chromatography. The final step yielded 20 mg of pure protein per liter of culture. The protein has a predicted molecular mass of 25440.6 Da which was confirmed by electrospray mass spectrometry ($M_{expt.}$ = 25439.9 ± 1.4 Da). The apparent K_m for CTP and NeuAc were determined to be 0.035 mM and 0.26 mM, respectively. As for the native enzyme, the recombinant enzyme was also capable of using N-glycolylneuraminic acid (NeuGc) as a substrate. Further experiments showed that the enzyme could accept

the deaminated neuraminic acid derivative 3-deoxy-D-glycero-D-galacto-nonulosonic acid (ketodeoxynonulosonic acid, KDN), as well.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Reactive Green 19-Agarose, Cibacron Blue 3GA-Agarose, CTP, N-acetylneuraminic acid, N-glycolylneuraminic acid, 2-keto-3-deoxy-D-manno-octulosonic acid, N-acetylneuraminic acid aldolase (*E. coli*), ampicillin, kanamycin, and SDS-PAGE molecular weight standards were from Sigma. Size exclusion chromatography standards were from Sigma and BioRad. Isopropyl β-D-thiogalactopyranoside (IPTG) was from BRL, Life Technologies, Inc.

3.2.2 Methods

3.2.2.1 *Growth of Bacteria*

E. coli strains were grown on Luria-Bertani (LB) agar plates and in LB broth along with the appropriate antibiotic (50 μg/ml ampicillin or kanamycin).

3.2.2.2 *Screening of the H. ducreyi Genomic DNA Library*

The N-terminal amino acid sequence was reverse-translated employing the CompuGene program (Barnes 1987). Two degenerate oligonucleotides were constructed (Table 3.1). Oligonucleotide 1, a 23-mer containing a single inosine at position 15 and a degeneracy of 108, corresponds to the reverse translation of amino acids 1-8. Oligonucleotide 2 corresponds to the reverse translation of amino acids 14-20 and is a 20-mer with a single inosine and a degeneracy of 48. Both oligonucleotides were prepared

by the Protein Chemistry Facility at Washington University School of Medicine. A λDASHII library of *H. ducreyi* 35000 DNA (Palmer and Munson 1995) was plated, plaques transferred to Hybond-N, and DNA was immobilized by UV cross linking (Stratalinker) employing standard methodologies. Oligonucleotide 1 was end-labeled with $\gamma^{32}P$ using T4 polynucleotide kinase and hybridization was performed overnight in 5X SSPE (1X SSPE = 0.18 M NaCl, 0.01 M sodium phosphate and 1 mM EDTA, pH 7.7), 5X Denhardt's solution, 0.5% sodium dodecyl sulfate (SDS) and 20 mg/ml Salmon testes DNA at 30°C. Following hybridization, the membrane was washed three times at 35°C in 2X SSPE-0.1% SDS. The membrane was then wrapped in Saran Wrap and autoradiographed. Phage plaques which gave a positive hybridization signal were picked and rescreened. Phage DNA was isolated from 10 ml liquid lysates and characterized by restriction analysis.

3.2.2.3 *Cloning and DNA Manipulations*

The low copy number vector pWSK30 was employed for the initial subcloning (Wang and Kushner 1991). For subsequent studies, the DNA fragment containing CMP-NeuAc synthetase was amplified by 20 rounds of PCR using primers 3 and 4 (Table 3.1). The PCR product was cloned with the TA cloning kit into pCRII according to the manufacturer's instructions (Invitrogen). Plasmid vectors pT7-7 (Tabor and Richardson 1985) and pET24b (Novagen) were employed for the preparation of the T7 expression constructs. Plasmid DNA was purified by using the Qiawell-8 plasmid kit (Qiagen, Chatsworth, CA).

DNA sequence was determined by the dideoxychain termination method employing Sequenase according to the manufacturer's directions (U.S. Biochemicals, Cleveland, OH). Lasergene software (DNASTAR, Madison, WI) was employed for contig assembly and sequence analysis.

Table 3.1. Oligonucleotides employed in this study

Oligonucleotide	Sequence [a]
1	5'ATGAARAARATHGCIATHATHCC
2	5'AARGGIATHAARGAYAARAA
3	5'AGATG**CATATG**AAGAAGATTGCAATCATC
4	5'CG**GGATCC**TGGCAATTTCTTTCATCGTTAT

[a] R=A/G, Y=C/T, H=T/C/A and I is inosine. The sequences in bold are the *Nde*I site in oligonucleotide 3 and the *Bam*HI site in oligonucleotide 4.

3.2.2.4 *Protein Sequence Alignment*

Protein sequence databases (GenBank, Release 92.0, Dec. 1995; SWISS-PROT, Release 32.0, Dec. 1995; PIR, Release 45.0, June 1995) were searched using the search program BLAST (Altschul et al. 1990) and protein sequences were aligned with the PILEUP program from the Wisconsin Sequence Analysis Package, Version 8.0 (Genetics Computer Group, Inc., Madison, WI). The similarity matrix was calculated using the scoring method in (Smith and Smith 1990). In pairwise comparisons, the smaller of the two sequences was taken as the denominator.

3.2.2.5 *Protein Expression*

E. coli BL21(DE3) harboring the pET24 expression construct containing the CMP-NeuAc synthetase (designated pMVT1) was grown at 30°C in LB broth containing 50 μg/ml kanamycin. After reaching an OD_{600} between 0.3 and 0.8, the culture was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) for 10 hrs at 30°C. The cells were harvested and frozen at -80°C until needed.

3.2.2.6 *Purification of Recombinant CMP-NeuAc Synthetase*

The cell paste (11 g) from a 3 L culture was suspended in 40 ml of 50 mM Tris-HCl, 0.1 M NaCl, pH 7.5 (buffer A) and the cells were lysed by two passages through a French press (approx. 20,000 psi). The lysate was sonicated (four 30 sec pulses at 25 watts with 2 min pauses) to shear nucleic acids and reduce its viscosity. The lysate was then centrifuged at 15,000 x g for 90 min to remove cellular debris. The supernatant (39 ml) was applied to a DEAE-cellulose column (2.5 cm x 13.5 cm) equilibrated with buffer A. After washing the column with 120 ml of buffer A the enzyme was eluted with a 270 ml gradient of 0.1 to 1.0 M NaCl. The active fractions were pooled (120 ml) and adjusted to 50% $(NH_4)_2SO_4$. The precipitate was pelleted, redissolved to 10 ml, and desalted into buffer A using a 15 ml Swift desalting column (Pierce). The sample was applied to a Reactive Green 19-Agarose dye column (2.5 cm x 14 cm) equilibrated with

buffer A. The column was washed with 120 ml of buffer A and most of the enzyme was then eluted with a step gradient of 50 mM Tris-HCl, 0.5 M NaCl, pH 7.5. The sample (25 ml) was adjusted to 50% $(NH_4)_2SO_4$ and the precipitate pelleted. The pellet was redissolved to 4.8 ml with 0.1 M KH_2PO_4, 0.5 mM EDTA, pH 7.0 (buffer B) and then saturated $(NH_4)_2SO_4$ (1.2 ml) was slowly added. The sample was centrifuged at 12,000 x g for 30 min to remove particulates before loading on the HIC column equilibrated with 0.1 M KH_2PO_4, 0.5 mM EDTA, 1.2 M $(NH_4)_2SO_4$, pH 7.0. The enzyme was eluted with a gradient of buffer B (1 ml/min, 1.2 - 0.6 M $(NH_4)_2SO_4$ in 40 min). Four separate runs were required to purify the entire sample. Fractions judged to be pure by SDS-PAGE were pooled (13.5 ml) and adjusted to 65% $(NH_4)_2SO_4$ for storage as a suspension at 4°C.

3.2.2.7 *CMP-NeuAc Synthetase Assay*

CMP-NeuAc synthetase activity was assayed by the following procedure: 10 µl of enzyme, diluted in 0.1 mg/ml bovine serum albumin (BSA), was added to 90 µl of assay mix and incubated at 37°C for 10 min. The reaction was stopped by adding 400 µl of cold water and freezing the sample in dry ice. The final concentrations of components in the assay mixture were 1 mM CTP, 2 mM NeuAc, 20 mM $MgCl_2$, 0.2 M MOPS, pH 7.1, 10 µg/ml BSA and 4-10 mU/ml (nmol/min/ml) CMP-NeuAc synthetase. The assay solutions were thawed directly before analysis. The anion exchange HPLC method discussed in Chapter 2 was used to quantitate the amount of CMP-NeuAc produced. A 20 µl loop was completely filled by a 100 µl injection. A unit is defined as the amount of enzyme that catalyzes the formation of one µmol of CMP-NeuAc per min under the conditions of the assay.

For calculation of the K_m for CTP and NeuAc, the enzyme was diluted with 0.2 M NaCl, 1 mM $MgCl_2$, 1 mg/ml BSA to 1 and 4 µg/ml, respectively. The diluted enzyme (35 µl), was added to 315 µl of assay mix preheated to 37°C and incubated at 37°C for up to 10.5 min. Each substrate was measured with the other held constant at a saturating concentration. NeuAc was varied from 0.25-8 mM with CTP kept at 1 mM and CTP was

varied from 0.025-0.8 mM with NeuAc at 10 mM. The final concentrations of the other components of the assay mixture were 20 mM $MgCl_2$, 20 mM NaCl, 0.2 M MOPS, pH 7.1, 0.1 mg/ml BSA and 0.1 or 0.4 µg/ml CMP-NeuAc synthetase. Aliquots (100 µl) were removed from the assays at 3.5 and 7 min and frozen in dry ice along with the final time point at 10.5 min. CMP-NeuAc product for each time point was quantitated as above, except that a 50 µl loop was filled with a 100 µl injection. A straight line was fit to the three time points and the slope was taken as the initial velocity. The K_m for CTP and NeuAc were determined by fitting the data directly to the equation $v = V_{max}[S]/(K_m + [S])$ using the computer program KinetAsyst (IntelliKinetics, State College, PA).

3.2.2.8 *Protein Determination*

Protein was determined using the BioRad DC Protein Assay (Alam 1992) according to the manufacturer's instructions. BSA was used as a standard.

3.2.2.9 *SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Analysis of chromatography fractions and calculation of molecular weight was performed using 15% acrylamide gels (Laemmli 1970). Gels were stained with Coomassie Brilliant Blue R-250. Molecular weight standards used were albumin, 66000; ovalbumin, 45000; glyceraldehyde-3-phosphate dehydrogenase, 36000; carbonic anhydrase, 29000; trypsinogen, 24000; trypsin inhibitor, 20000; α-lactalbumin, 14200; and aprotinin, 6500.

3.2.2.10 *Reverse Phase Chromatography*

Purified CMP-NeuAc synthetase (10 µg) from the HIC column was injected on to a C4 reverse phase column (Vydac; 2.1 x 150 mm) equilibrated with 0.1% TFA. The enzyme was eluted with a gradient of 0.08% TFA, 70% CH_3CN over 70 min at a flow rate of 0.2 ml/min. Detection was at 215 nm.

3.2.2.11 *Size Exclusion Chromatography*

To estimate the native molecular weight, purified enzyme (25-200 μg) was applied to two Bio-Silect SEC 250-5 columns (BioRad; 7.8 x 300 mm each) connected in series and equilibrated with 0.1 M KH_2PO_4, 0.15 M NaCl, pH 7.0. Elution was at 1 ml/min. The column was calibrated with molecular weight standards from BioRad (thyroglobulin, 670000; IgG, 158000; ovalbumin, 44000; myoglobin, 17500) and Sigma (albumin, 66000; carbonic anhydrase, 29000; cytochrome c, 12400; aprotinin, 6500), both run separately from each other and the CMP-NeuAc synthetase. The CMP-NeuAc synthetase was exchanged into the column buffer with a 15 ml Swift desalting column, desalted with water using a Microcon-10 microconcentrator (Amicon), or resuspended from an ammonium sulfate pellet with the column buffer prior to chromatography.

3.2.2.12 *Electrospray Mass Spectrometry*

The CMP-NeuAc synthetase was desalted by gradient elution from a Vydac C4 reverse phase HPLC column or by repeated concentration and dilution with water using a Microcon-10 microconcentrator. A 5 μl aliquot (approx. 5 μg) of this solution was injected into a stream of H_2O/CH_3CN (1:1) (with or without 1% acetic acid) at a flow rate of 20 μl/min which was coupled to the electrospray ionization (ESI) source of a Fison Platform quadrupole mass spectrometer (Fison, Manchester U.K.). The mass spectrometer was run in the positive-ion mode and scanned from *m/z* 600 to 2000 in 8.1 sec intervals. Horse heart myoglobin (average M_r = 16951.5) was used as a reference compound and external calibrant and a typical mass accuracy of ± 0.01% was obtained.

3.2.2.13 *Substrate Specificity*

To determine whether the CMP-NeuAc synthetase could use sialic acids other than NeuAc as substrates, the enzyme was incubated with NeuGc or KDN. Conditions were chosen that favored complete conversion of CTP to the CMP-sialic acid compound. For the NeuGc reaction, 1 mM CTP, 1 mM NeuGc, 20 mM $MgCl_2$, 0.1 M MOPS, pH

7.8, and 100 µg CMP-NeuAc synthetase (1.3 U) in a volume of 100 µl was incubated at 37°C for 20 min. For the KDN reaction, KDN was generated in situ using NeuAc aldolase, pyruvate, and mannose. The reaction conditions were 1 mM CTP, 5 mM mannose, 5 mM pyruvate, 20 mM $MgCl_2$, 0.1 M MOPS, pH 7.8, 5 µl NeuAc aldolase (8 µg, 0.17 U), and 40 µg CMP-NeuAc synthetase (0.5 U) in a volume of 100 µl. The reaction was incubated at 37°C for 90 min. An identical reaction was conducted as a control except that ManNAc replaced mannose so that CMP-NeuAc would be formed as the final product. All three reactions were subjected to anion exchange to purify the CMP-sialic acid products using NH_4HCO_2 (pH not adjusted). The products were, lyophilized, redissolved with 5 ml H_2O, and relyophilized. After redissolving in 50 µl H_2O the samples were analyzed by positive-mode ESI-MS by injecting 5 µl into a stream of H_2O/CH_3CN (1:1) with 1% AcOH as described above for analysis of the enzyme.

3.3 RESULTS AND DISCUSSION

In order to clone the gene for the *H. ducreyi* CMP-NeuAc synthetase, the amino terminal sequence was reverse translated and two degenerate oligonucleotides were constructed (Table 3.1). Approximately 1200 clones from a λDASHII library of strain 35000 DNA were screened with oligonucleotide 1 and 7 hybridization-positive clones were identified. After plaque purification and rescreening, DNA was prepared from each clone and characterized by restriction analysis and by Southern hybridization. Two of the clones were identical and the additional 5 clones had overlapping restriction maps. One clone was chosen for further characterization. *H. ducreyi* DNA fragments of 5.5 Kb and 8 Kb were identified in a *Not*I digest, cloned into *Not*I-digested pWSK30 and the mixture was transformed into *E. coli* DH5α. Clones containing either the 5.5 Kb or the 8 Kb fragment were identified. Southern analysis using oligonucleotides 1 and 2 indicated that the *H. ducreyi* CMP-NeuAc synthetase gene was localized to the 8 Kb fragment. A

clone containing the 8 Kb *Not*I fragment, designated pRSM1627, was saved. Oligonucleotide 1 was used as a sequencing primer and sequence was determined with Sequenase. Additional primers were constructed and the complete DNA sequence of the *H. ducreyi* CMP-NeuAc synthetase gene (*neuA*) was determined in both directions. The position and direction of transcription of the *H. ducreyi* CMP-NeuAc synthetase gene in pRSM1627 was determined by PCR analysis (Fig. 3.1).

An open reading frame of 684 bp encoding a 228 residue protein was identified (Fig. 3.2). An inverted repeat characteristic of a rho-independent transcriptional terminator was identified 63 nucleotides downstream of the termination codon. Putative promoter sequences were not identified.

A T7 expression system was employed to generate large quantities of the *H. ducreyi* CMP-NeuAc synthetase. The gene was first amplified by PCR and recloned by TA cloning. The 5' PCR primer contained an *Nde*I site (Table 3.1, oligonucleotide 3) and the 3' primer contained a *Bam*HI site (Table 3.1, oligonucleotide 4). The sequence of the clone in the TA vector was again determined to verify that there were no PCR errors. The CMP-NeuAc synthetase gene was initially cloned as a *Nde*I-*Bam*HI fragment into pT7-7 which had been digested with the same enzymes. The construct was readily transformed into *E. coli* strains lacking a source of T7 polymerase, however, no transformants were obtained with competent BL21(DE3) cells suggesting that the synthetase gene product was being expressed and was toxic to the cells. Transformants were obtained after cloning the gene as a *Nde*I-*Bam*HI fragment into the more tightly regulated T7lac pET24 vector. Initial expression experiments at 37°C and inducing with 1 mM IPTG showed the synthetase to be the most abundant protein in cell lysates and enzyme activity levels in a crude lysate to be several hundred times higher than from *H. ducreyi* lysates. However, a large portion of the enzyme (approx. 75%) was found in the insoluble cell pellet. Lower temperatures (20-30°C) and IPTG concentration (0.1 mM)

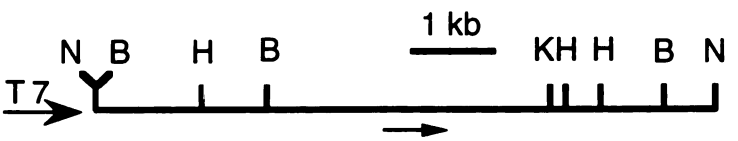


Fig. 3.1. **Partial restriction map of pRSM1627**. An 8 kb NotI fragment containing the *H. ducreyi neuA* gene was subcloned from a lambda clone into pWSK30. The restriction site designations are: N, *Not*I; B, *Bam*HI; H, *Hind*III; and K, *Kpn*I. The *H. ducreyi neuA* gene was localized on the NotI fragment by PCR analysis. The arrow designates the position of the gene and direction of transcription.

Fig. 3.2. **Nucleotide and derived amino acid sequence of the *H. ducreyi neuA* gene.** The DNA sequence of the *H. ducreyi neuA* gene was determined on both strands of pRSM1627. The initial sequence was determined with the degenerate oligonucleotide 1 as a sequencing primer and the remainder of the sequence was determined by walking, using primers synthesized as needed using the previously determined sequence. Amino acids 1 through 43 (in bold typeface) correspond to the sequence determined by Edman degradation. Underlined nucleotides correspond to an inverted repeat characteristic of a rho-independent transcriptional terminator.

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TAACTTTTTTTGCTGCTGCGTCTGTAAAGGTAAGAGGAATTTGATCGATATCGCTCATTC    60

AATCTTCCCACATAAAAATCAGATCATTTTGCCATTATCTACCAATCTGATTGGTTGAGC   120

AATAGCATTTATGATAAAGATTATTGTCTATAAGATTGTAAGAATGTTTAAATGTTATAC   180

ATTAAAATGCAATAAACGGACTATTCATAGTATAGTGGCGTTATTTTATGTACCATAGTG   240

GTTACTTTTTATTAATTATACATTGATAGATGACGATGAAGAAGATTGCAATCATCCCTG   300
                                  M  K  K  I  A  I  I  P

CCCGTGCCGGCTCAAAAGGTATCAAGGATAAAAAACCTACAATTAGTTGGCGGTATTTCAT   360
A  R  A  G  S  K  G  I  K  D  K  N  L  Q  L  V  G  G  I  S

TGGTTGGTCGAGCGATTATCGCCGCTCAGCAAGCGGGTATTTTTGATCATATTATTGTCA   420
L  V  G  R  A  I  I  A  A  Q  Q  A  G  I  F  D  H  I  I  V

GTTCGGATGGCGATCATATTTTAAAAGAGGCGGAACGTTATCAAGCAGAAACATTAAAGC   480
S  S  D  G  D  H  I  L  K  E  A  E  R  Y  Q  A  E  T  L  K

GACCTTCTTATTTAGCGCAAAGTGAGACAAAAAGCATTGATGTGATCCTTCACGCTTTGG   540
R  P  S  Y  L  A  Q  S  E  T  K  S  I  D  V  I  L  H  A  L

AGGCATTACAGTTTAGCGATGGAATAGTGGTACTTATCCAACCAACTAGCCCGTTGCGAT   600
E  A  L  Q  F  S  D  G  I  V  V  L  I  Q  P  T  S  P  L  R

CAGAGATTGATATTAAAAACGCGATGGCACTTTTTTTAGAAGGGAAGTATCACGCTGTAA   660
S  E  I  D  I  K  N  A  M  A  L  F  L  E  G  K  Y  H  A  V

TTTCTGCTTGTGAATGTGAACATCATCCTTATAAAAGTTTTTTATTAGAACAGGATAATA   720
I  S  A  C  E  C  E  H  H  P  Y  K  S  F  L  L  E  Q  D  N

TTATTCCATTAACAGATATCAACGATTTTGAAGTGCCACGACAAAAATTACCGAAATCCT   780
I  I  P  L  T  D  I  N  D  F  E  V  P  R  Q  K  L  P  K  S

ATCGGGCAAATGGAGCGATTTATATCAATGATATTGCAGCACTTATTCAACAAAAAAGAT   840
Y  R  A  N  G  A  I  Y  I  N  D  I  A  A  L  I  Q  Q  K  R

TTTTTGTTGAACCGATGCAGTTCTATTTGATGCCACAAGATCGTTCGATTGATATTGATG   900
F  F  V  E  P  M  Q  F  Y  L  M  P  Q  D  R  S  I  D  I  D

CAATTATTGATCTTAAAATGGCAGAGTTATTGCTACAACATGAGCATGCCATTATTTAGT   960
A  I  I  D  L  K  M  A  E  L  L  L  Q  H  E  H  A  I  I  *

TTAATACTAAATTTAATTAAAACGGATTATTTTTGGATCAAATAACGATGAAAGAAATTG  1020

CCATTATTAGCAATGCAAAGAATGTTTTTTTTATATTGTTTACTGACTAATAAAAATGTA  1080
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favored more soluble enzyme, although insoluble CMP-NeuAc synthetase was still found in the lysate pellet under the best conditions identified.

The enzyme was the major component of the soluble cell lysate. This greatly simplified the purification and allowed a homogeneous preparation of enzyme to be obtained in only three steps. For this, successive anion exchange, Green 19 dye, and hydrophobic interaction chromatography steps were used and 20 mg of pure protein per liter of culture was obtained. In the final purification step a single, large peak was eluted from an HIC column using a shallow gradient (23.5 column volumes over 40 min) (Fig. 3.3). A summary of the purification of the recombinant enzyme is given in Table 3.2 and the SDS-PAGE analysis of the pooled fractions from each chromatography step is shown in Fig. 3.4. The enzyme appeared highly pure by SDS-PAGE (lane D, Fig. 3.4) even though the gel was overloaded. The enzyme also appeared homogeneous by reverse phase chromatography (Fig. 3.5). A molecular mass of 25.6 kDa was determined by SDS-PAGE which is very close to the calculated average mass of 25440.6 Da (Fig. 3.6). The calculated average mass was confirmed by electrospray mass spectrometry with an accuracy to within ± 1.4 Da (Fig. 3.7). By size exclusion chromatography the enzyme's molecular mass was calculated as 47-74 kDa in 0.1 M KH_2PO_4, 0.15 M NaCl, pH 7.0 buffer and thus appears to exist as a dimer or possibly larger species. The concentration of enzyme affected the retention time, with higher concentrations shifting the peak to earlier retention times (higher molecular mass). Whether the enzyme was resuspended from an ammonium sulfate suspension, or desalted with the column buffer or water, most of the enzyme eluted as a higher molecular weight species with little evidence for the presence of a monomeric form. However, if the enzyme was left at 4°C overnight in water or column buffer, both the larger species and a monomeric species were detected by size exclusion chromatography (Fig. 3.8). Incubation overnight at 4°C in 0.2 M NaCl (no phosphate buffer) resulted in very little of the monomeric species. The retention time of the monomer showed little dependence on enzyme concentration. The rat liver CMP-NeuAc synthetase was identified as a dimer by size exclusion

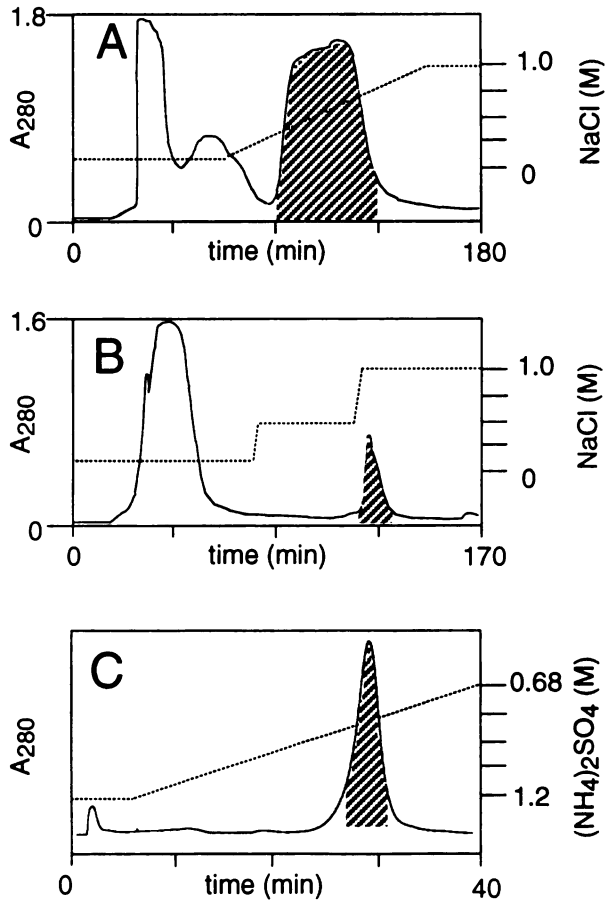


Fig. 3.3. **Purification of the recombinant CMP-NeuAc synthetase.** (A) Anion exchange chromatography of the *E. coli* lysate using a DEAE-cellulose column (2.5 x 13.5 cm) operated at 3 ml/min. Fractions containing enzyme activity are shaded. (B) The active fractions from (A) were pooled, adjusted to 50% $(NH_4)_2SO_4$, and the CMP-NeuAc synthetase activity pelleted. After desalting, the sample was applied to a Green 19 dye column (2.5 x 14 cm) operated at 2 ml/min and CMP-NeuAc synthetase eluted with 0.5 M NaCl. Active fractions are shaded. (C) The enzyme eluted from the Green 19 column was precipitated with 50% $(NH_4)_2SO_4$, and the pellet dissolved in the minimum volume prior to loading on the hydrophobic interaction column. Fractions were analyzed by SDS-PAGE for purity and those deemed homogeneous were pooled (shaded).

Table 3.2. Purification summary of the recombinant *H. ducreyi* CMP-NeuAc synthetase

Chromatography step	Protein	Units	Specific Activity	Purification	Recovery
	mg	*μmol/min*	*μmol/min/mg*	*fold*	*% activity*
Lysate	718	3400	4.74	1	100
DEAE-cellulose	372	2222	5.97	1.26	65
Green 19	110	1382	12.56	2.65	41
HIC	61	772	12.66	2.67	23

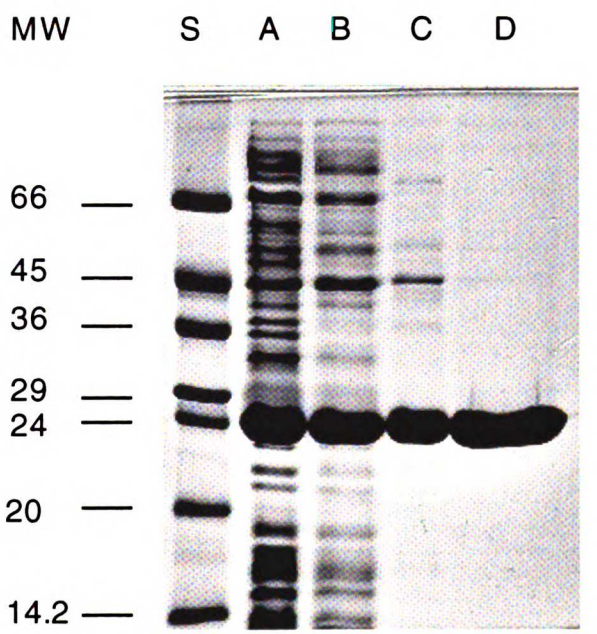


Fig. 3.4. **SDS-PAGE analysis of fractions from the purification of the recombinant CMP-NeuAc synthetase**. Samples from each of the purification steps were loaded on a 15% SDS-polyacrylamide gel and stained with Coomassie Blue after electrophoresis. Lanes: (S), molecular weight standards; (A), lysate; (B), DEAE-cellulose pool; (C), Green 19 pool; (D), HIC pool (18 µg CMP-NeuAc synthetase). The numbers to the left refer to the molecular mass of the standards in kilodaltons.

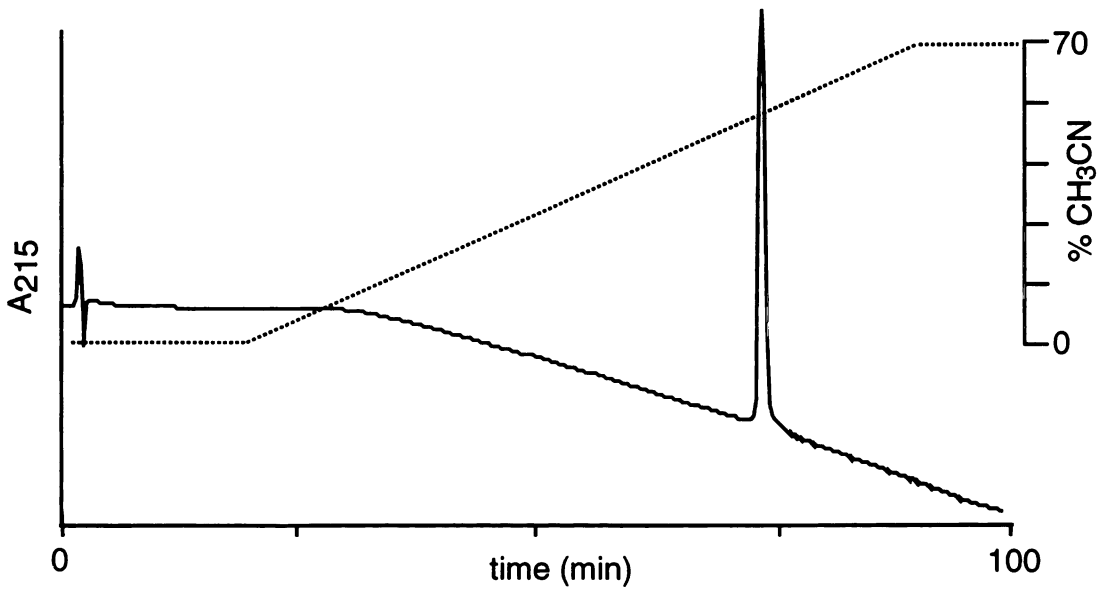


Fig. 3.5. **Reverse phase chromatography of the recombinant CMP-NeuAc synthetase.** Purified enzyme (10 μg) from the HIC column was injected on to a C4 reverse phase column (Vydac, 2.1 x 150 mm) and eluted with a gradient of CH_3CN. A single peak was eluted from the column at 74.3 min demonstrating the high purity obtained by the enzyme purification procedure (Fig. 3.3).

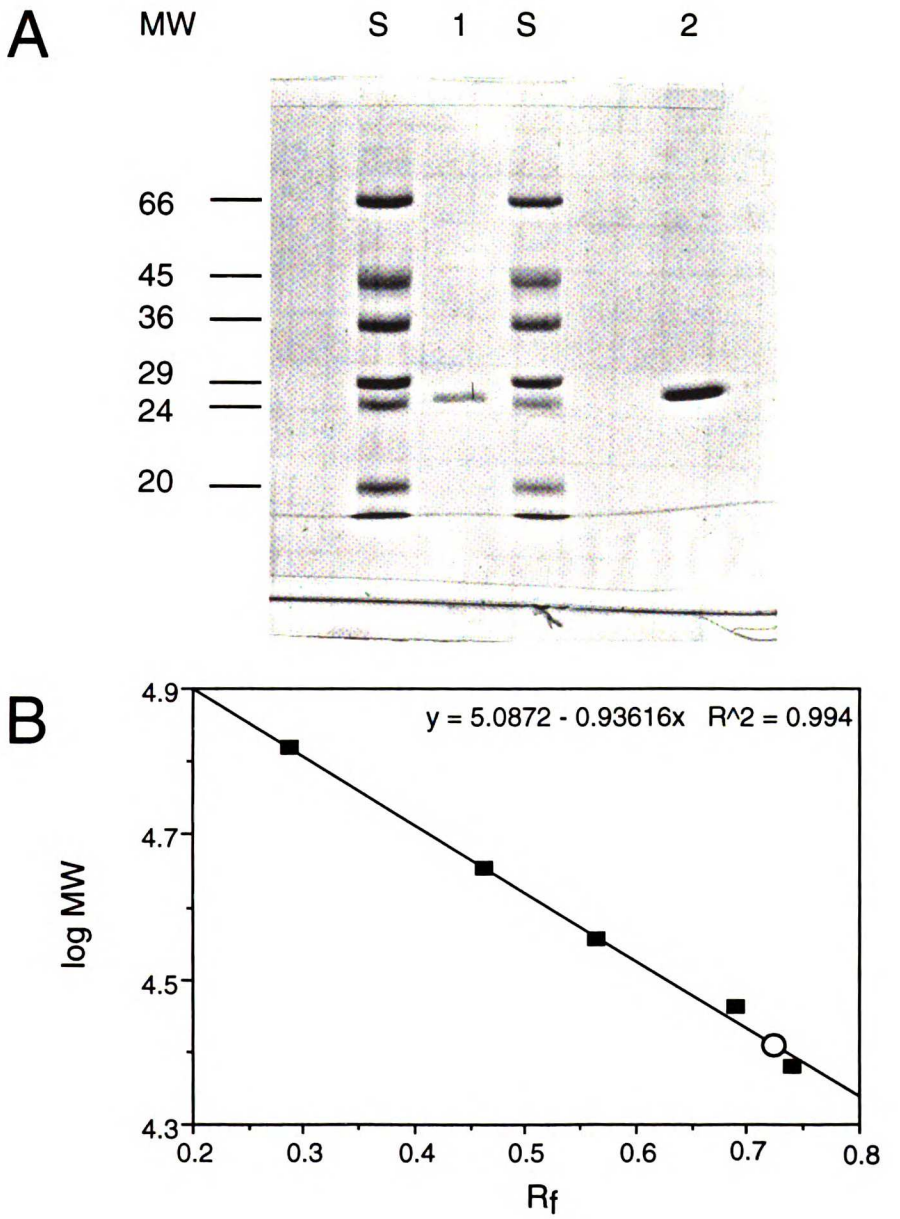


Fig. 3.6. **SDS-PAGE for the calculation of the recombinant CMP-NeuAc synthetase molecular mass.** (A) Purified enzyme and molecular weight standards were loaded on a 15% SDS-polyacrylamide gel and stained with Coomassie blue after electrophoresis. Lanes: (S), molecular weight standards; (1) and (2), CMP-NeuAc synthetase. The numbers to the left refer to the molecular mass of the standards in kilodaltons. (B) Semilog plot of molecular mass versus the relative distance migrated. Only standards from 66 kDa to 24 kDa were used for the plot. The circle indicates where the CMP-NeuAc synthetase would fall on the line.

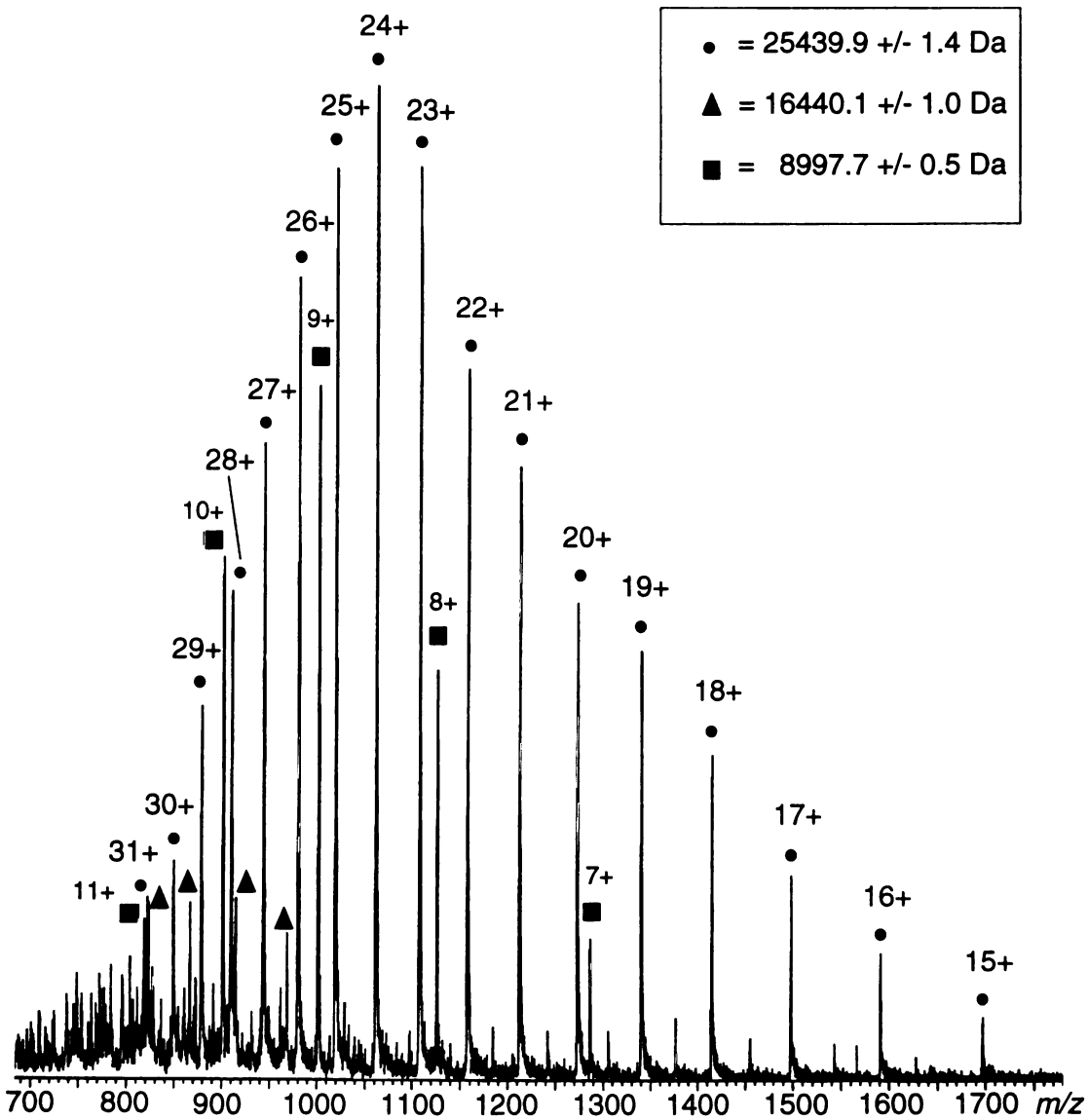


Fig. 3.7. **Electrospray mass spectrum of the recombinant CMP-NeuAc synthetase.** The mass of the enzyme (25439.9 ± 1.4 Da) was within experimental error of the predicted average molecular mass (25440.6 Da). In addition to the intact protein, two fragments (16440.1 + 8997.7 = 25437.8 Da) were detected. These species arose from a gas-phase fragmentation between residues Ile[150] and Pro[151] and not chemical hydrolysis i.e., no addition of H_2O was found in the N-terminal fragment and the percentage of fragmentation was highly dependent on the cone voltage.

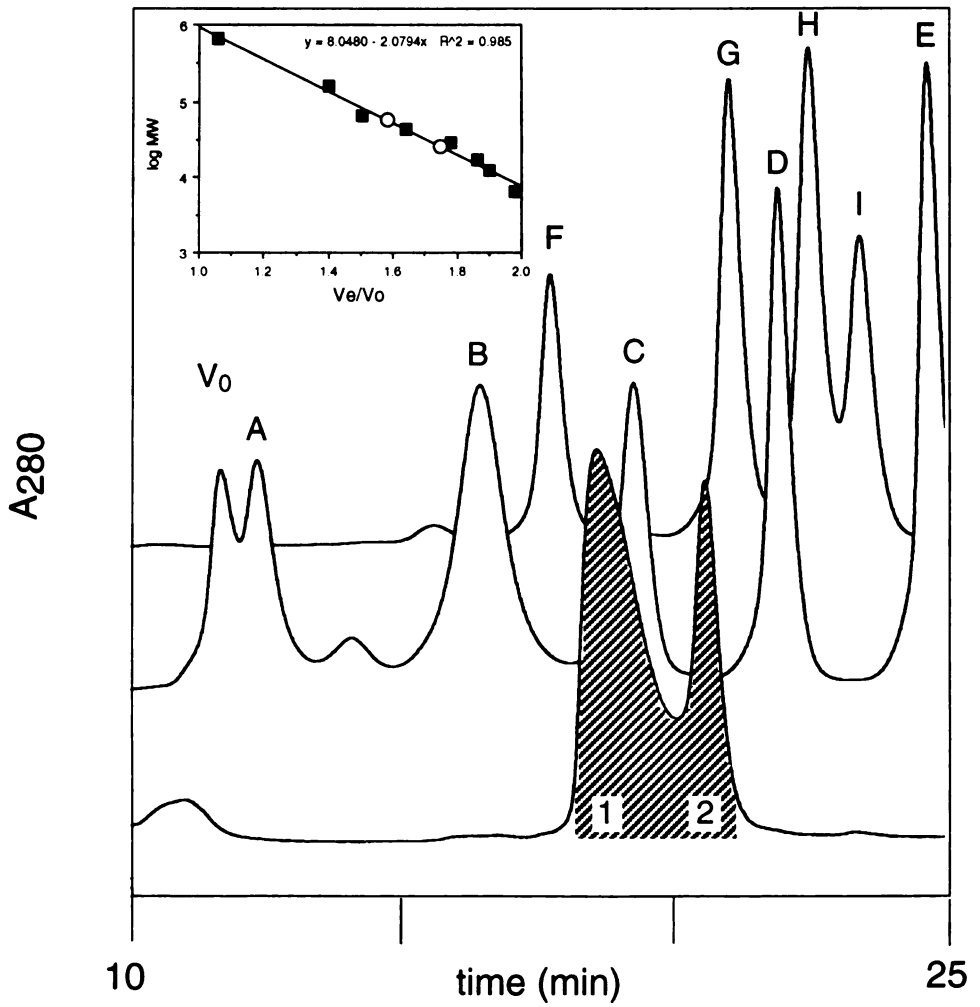


Fig. 3.8. **Size exclusion chromatography of the recombinant CMP-NeuAc synthetase.** Molecular weight profile of CMP-NeuAc synthetase (1 and 2) and molecular weight standards (A-I). The elution time of peak (1) was dependent on enzyme concentration giving a calculated molecular mass ranging from 47 to 74 kDa. The monomer (2) always eluted over a much narrower range of 26 to 28 kDa. (Inset) Semilog plot of molecular mass versus V/V_0 (V_e = elution volume, V_0 = void volume) See "Methods" for the molecular weight standards used (A-E, BioRad; F-I, Sigma). Standard E (Vitamin B-12, 1350 Da) was not used in constructing the calibration. The circles indicate where the CMP-NeuAc synthetase would fall on the line. The calculated molecular mass for peak (1) is 58 kDa and for peak (2) is 26 kDa.

chromatography (Rodriguez-Aparicio et al. 1992). The *E. coli* enzyme is thought to be active as a monomer, but did form dimers and larger aggregates in some buffers (Zapata et al. 1989). The *Neisseria meningitidis* enzyme also possibly exists as a dimer based on size exclusion chromatography results (Ganguli et al. 1994). The active form of the *H. ducreyi* enzyme and the exact number of subunits that exist in the larger species remains to be determined. The apparent K_m for CTP and NeuAc were found to be 0.035 mM and 0.26 mM (mean of three experiments), respectively, at pH 7.1 (Fig. 3.9). The K_m for NeuAc is similar to that measured for the *N. meningitidis* enzyme but the K_m for CTP is much lower than values obtained for previously described activities (see (Haft and Wessels 1994) for review). A possible reason for such a large difference is that most other CMP-NeuAc synthetases have been assayed at a higher pH where activity is greater while the *H. ducreyi* enzyme was assayed at a more physiological pH. As for the native enzyme (see Chapter 2), the recombinant enzyme was also capable of using N-glycolylneuraminic acid (NeuGc) as a substrate. Another interesting discovery was that KDN could be used as a substrate. The reactions consumed all of the CTP present, generating products that eluted at the same position as CMP-NeuAc by anion exchange chromatography (data not shown). The purified CMP-sialic acid products were confirmed by ESI-MS (data not shown). KDN has a hydroxyl at the 5 position of neuraminic acid instead of an N-acetyl group. This suggests that the enzyme's recognition at this site is not as specific as the other characterized bacterial CMP-NeuAc synthetases.

There are now five sequences in the sequence databases identified as CMP-NeuAc synthetases. A sixth sequence from *Campylobacter coli* is not identified as such, but is highly similar (28% identical to the *H. ducreyi* enzyme) (ptmB, U25992; identified as being involved in the posttranslational modification of flagellin). All six sequences are from bacteria. Twenty residues from the N-terminus of the rat liver enzyme are known, but the sequence is not similar to any of the bacterial sequences. The *H. ducreyi* CMP-NeuAc synthetase is most similar to the *Haemophilus influenzae* and *N. meningitidis*

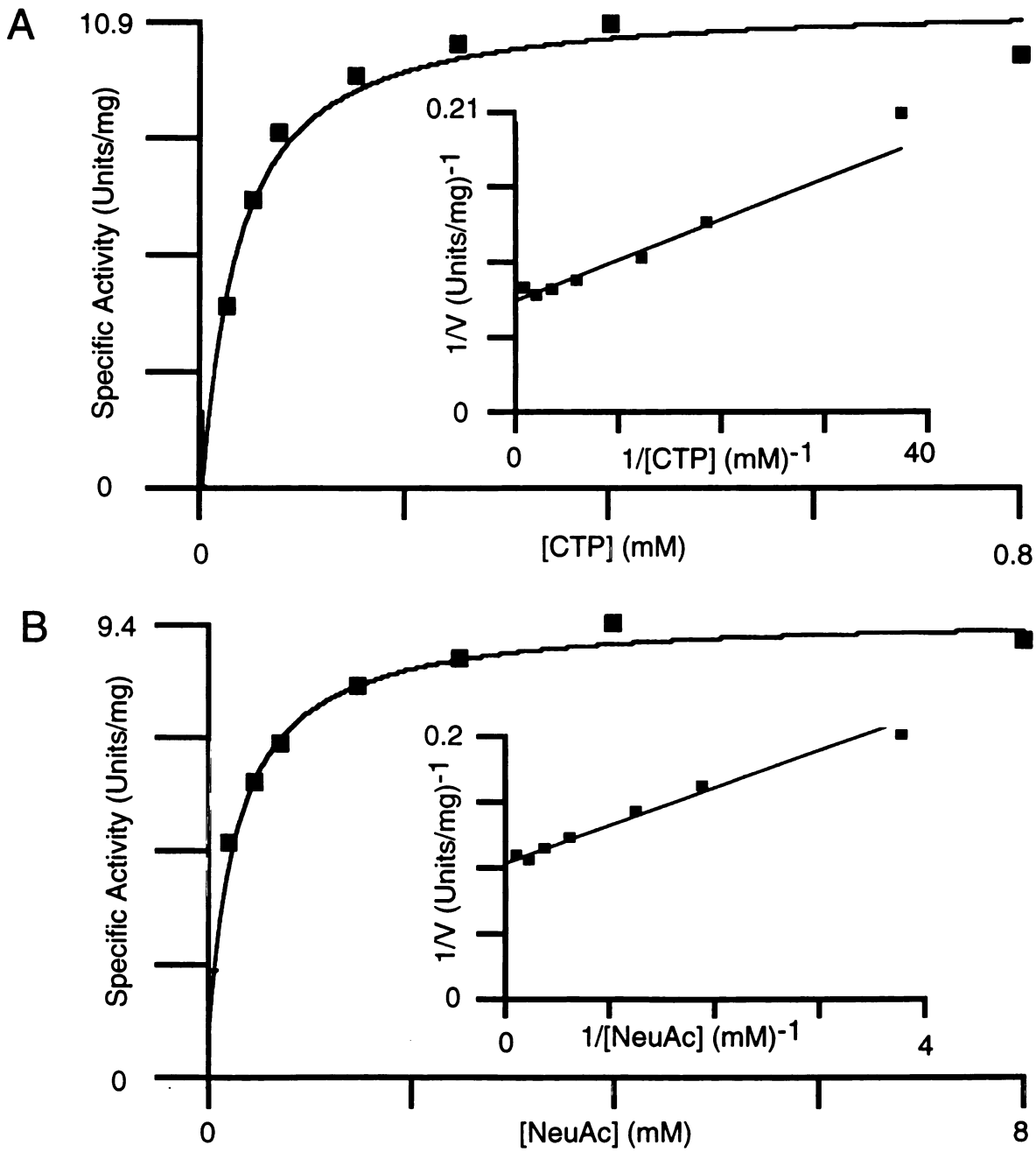


Fig. 3.9. **Effect of substrate concentration on the CMP-NeuAc synthetase reaction.** Determination of K_m for CTP (A) and NeuAc (B) using a saturating concentration of the other substrate. The K_m values were calculated by direct fit to the hyperbola.

Table 3.3. Similarity matrix for CMP-NeuAc and CMP-KDO synthetase proteins

			CMP-NeuAc synthetases					CMP-KDO synthetases			
	Protein	Accession Number	1	2	3	4	5	6	7	8	9
1	HD[a] NeuA	U54496		65[b] (79)	45 (62)	32 (51)	32 (50)	13 (37)	14 (35)	14 (34)	16 (35)
2	HI SiaB	C64114			46 (63)	33 (53)	34 (50)	13 (35)	11 (33)	13 (34)	15 (33)
3	NM SynB	X78068				34 (54)	35 (53)	17 (37)	18 (36)	14 (33)	17 (36)
4	EC NeuA	P13266					35[c] (51)	16 (39)	15 (39)	15 (37)	15 (37)
5	SA CpsF	U19899						18 (38)	17 (36)	15 (38)	14 (32)
6	EC KdsB	A26322							65 (79)	46 (62)	36 (55)
7	HI KdsB	G64045								45 (62)	37 (56)
8	EC KpsU	P42216									38 (58)
9	CT KdsB	U15192									

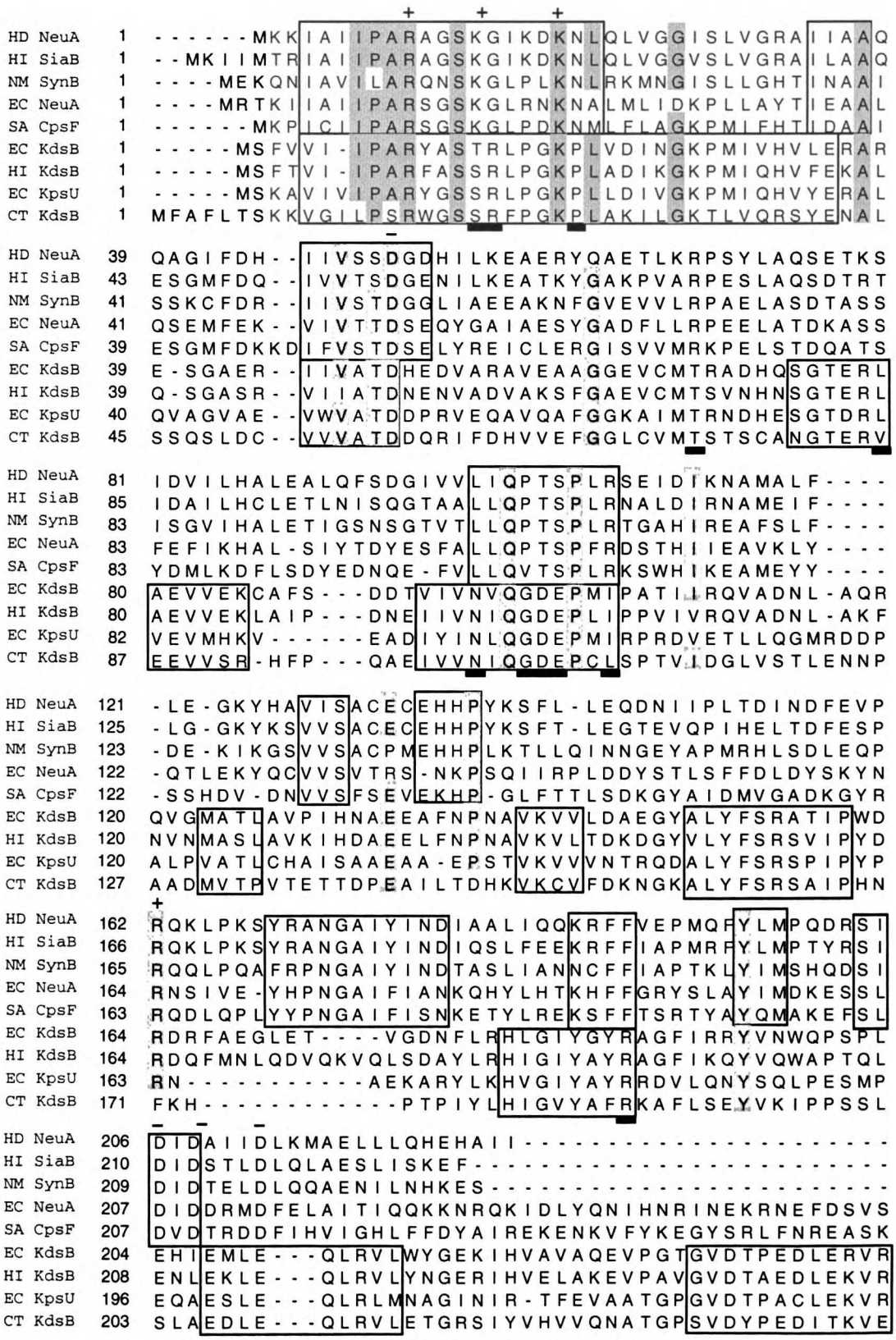
[a] The abbreviations used are the same as in Fig. 3.10.

[b] The percentage of identical residues is given for each pair of proteins from the sequence alignment shown in Fig. 3.10. The percentage of identical plus similar residues is given in parentheses. As a class, the CMP-NeuAc synthetases are 32-65% identical and share 50-79% similarity. Likewise, the CMP-KDO synthetases are 37-65% identical and share 55-79% similarity. The two classes are 11-18% identical and 32-39% similar.

[c] The values for the *E. coli* K1 NeuA and the *S. agalactiae* CpsF sequence comparison are for residues 1-261. When the entire proteins were compared the values were lowered to 27% identity and 44% similarity due to less similarity in their C-terminal region.

sequences being 65 and 45% identical (79 and 62% similar), respectively (Table 3.3). These three enzymes all have nearly the same molecular weight while the *E. coli* and *Streptococcus agalactiae* enzymes are nearly twice as large. The *H. ducreyi* CMP-NeuAc synthetase is 32% identical with the N-terminal half of the *E. coli* and *S. agalactiae* enzymes (see Table 3.3). Taken as a whole, the CMP-NeuAc synthetases have 32-65% identity and 50-79% similarity with each other. The CMP-NeuAc synthetases also have limited similarity to the four known CMP-KDO synthetase sequences, primarily in the N-terminal region (11-18% identity, 32-39% similarity). Although, the specificity of these enzymes for their sugar substrate is high, they catalyze a very similar reaction in which CMP is transferred from CTP to either NeuAc or KDO; both of which are α-keto acid sugars (Kohlbrenner and Fesik 1985; Kohlbrenner et al. 1987; Ambrose et al. 1992). These proteins do not contain the HiGH motif that is involved in nucleotide binding in the recently elucidated cytidylyltransferase superfamily (Bork et al. 1995). The highest similarity is found in the N-terminal region. The two previously noted regions of highest similarity between the *E. coli* NeuA and KdsB proteins largely holds true for the larger set of sequences as well (residues 6-10 and 46-51 in *H. ducreyi* NeuA) (Zapata et al. 1989). There are only a few residues conserved throughout in these two enzyme classes and these are likely to be involved in catalysis and binding of the common substrate, CTP (Fig. 3.10). A conserved arginine and lysine are found nine residues apart in the N-terminus along with a third arginine or lysine in between. Chemical modification and site-directed mutagenesis of the *E. coli* CMP-NeuAc synthetase suggests that arginine and lysine are important for enzyme activity (Vann et al. 1993; Zapata et al. 1995). In addition, there is almost a complete lack of negatively charged amino acids in this N-terminal region. There is also a third region of high similarity with a conserved glutamine, proceeded by several hydrophobic residues, and a conserved proline four residues apart (residues 98-107 in *H. ducreyi* NeuA). The residues in between are conserved within each class, but differ between the two classes ((P/V)TS and GDE for CMP-NeuAc and CMP-KDO synthetases respectively).

Fig. 3.10. **Sequence alignment of CMP-NeuAc synthetases and CMP-KDO synthetases.** Residues conserved (7 or more identical) across both enzyme classes are shaded. Conserved positive and negative charges are indicated by (+) and (-) signs above the residues. In two cases the conserved charged residues differ by one position between the two classes so the sign is placed between the residues. Boxes indicate areas of high percent identity among each class of enzymes. The large bars underline residues that are conserved within each class, but differ between the two. The abbreviation used are: HD, *H. ducreyi*; HI, *H. influenzae*; NM, *N. meningitidis*; EC, *E. coli*; SA, *S. agalactiae*; CT, *Chlamydia trachomatis.*


HD NeuA 1 ------MKK IAIIPARAGSKGIKDKNLQLVGGISLVGRAIIAAQ
HI SiaB 1 --MKIIMTR IAIIPARAGSKGIKDKNLQLVGGVSLVGRAILAAQ
NM SynB 1 ----MEKQN IAVILARQNSKGLPLKNLRKMNGISLLGHTINAAI
EC NeuA 1 ----MRTKI IAIIPARSGSKGLRNKNALMLIDKPLLAYTIEAAL
SA CpsF 1 ------MKP ICIIPARSGSKGLPDKNMLFLAGKPMIFHTIDAAI
EC KdsB 1 -----MSFV VI-IPARYASTRLPGKPLVDINGKPMIVHVLERAR
HI KdsB 1 -----MSFT VI-IPARFASSRLPGKPLADIKGKPMIQHVFEKAL
EC KpsU 1 -----MSKA VIVIPARYGSSRLPGKPLLDIVGKPMIQHVYERAL
CT KdsB 1 MFAFLTSKK VGILPSRWGSSRFPGKPLAKILGKTLVQRSYENAL
HD NeuA 39 QAGIFDH--IIVSSDGDHILKEAERYQAETLKRPSYLAQSETKS
HI SiaB 43 ESGMFDQ--IVVTSDGENILKEATKYGAKPVARPESLAQSDTRT
NM SynB 41 SSKCFDR--IIVSTDGGLIAEEAKNFGVEVVLRPAELASDTASS
EC NeuA 41 QSEMFEK--VIVTTDSEQYGAIAESYGADFLLRPEELATDKASS
SA CpsF 39 ESGMFDKKDIFVSTDSELYREICLERGISVVMRKPELSTDQATS
EC KdsB 39 E-SGAER--IIVATDHEDVARAVEAAGGEVCMTRADHQSGTERL
HI KdsB 39 Q-SGASR--VIIATDNENVADVAKSFGAEVCMTSVNHNSGTERL
EC KpsU 40 QVAGVAE--VWVATDDPRVEQAVQAFGGKAIMTRNDHESGTDRL
CT KdsB 45 SSQSLDC--VVVATDDQRIFDHVVEFGGLCVMTSTSCANGTERV
HD NeuA 81 IDVILHALEALQFSDGIVVLIQPTSPLRSEIDIKNAMALF----
HI SiaB 85 IDAILHCLETLNISQGGTAALLQPTSPLRNALDIRNAMEIF----
NM SynB 83 ISGVIHALETIGSNSGTVTLLQPTSPLRTGAHIREAFSLF----
EC NeuA 83 FEFIKHAL-SIYTDYESFALLQPTSPFRDSTHIIEAVKLY----
SA CpsF 83 YDMLKDFLSDYEDNQE-FVLLQVTSPLRKSWHIKEAMEYY----
EC KdsB 80 AEVVEKCAFS---DDTVIVNVQGDEPMIPATIIRQVADNL-AQR
HI KdsB 80 AEVVEKLAIP---DNEIIVNIQGDEPLIPPVIVRQVADNL-AKF
EC KpsU 82 VEVMHKV------EADIYINLQGDEPMIRPRDVETLLQGMRDDP
CT KdsB 87 EEVVSR-HFP---QAEIVVNIQGDEPCLSPTVIDGLVSTLENNP
HD NeuA 121 -LE-GKYHAVISACECEHHPYKSFL-LEQDNIIPLTDINDFEVP
HI SiaB 125 -LG-GKYKSVVSACECEHHPYKSFT-LEGTEVQPIHELTDFESP
NM SynB 123 -DE-KIKGSVVSACPMEHHPLKTLLQINNGEYAPMRHLSDLEQP
EC NeuA 122 -QTLEKYQCVVSVTRS-NKPSQIIRPLDDYSTLSFFDLDYSKYN
SA CpsF 122 -SSHDV-DNVVSFSEVEKHP-GLFTTLSDKGYAIDMVGADKGYR
EC KdsB 120 QVGMATLAVPIHNAEEAFNPNAVKVVLDAEGYALYFSRATIPWD
HI KdsB 120 NVNMASLAVKIHDAEELFNPNAVKVLTDKDGYVLYFSRSVIPYD
EC KpsU 120 ALPVATLCHAISAAEAA-EPSTVKVVVNTRQDALYFSRSPIPYP
CT KdsB 127 AADMVTPVTETTDPEAILTDHKVKCVFDKNGKALYFSRSAIPHN
HD NeuA 162 RQKLPKSYRANGAIYINDIAALIQQKRFFVEPMQFYLMPQDRSI
HI SiaB 166 RQKLPKSYRANGAIYINDIQSLFEEKRFFIAPMRFYLMPTYRSI
NM SynB 165 RQQLPQAFRPNGAIYINDTASLIANNCFFIAPTKLYIMSHQDSI
EC NeuA 164 RNSIVE-YHPNGAIFIANKQHYLHTKHFFGRYSLAYIMDKESSL
SA CpsF 163 RQDLQPLYYPNGAIFISNKETYLREKSFFTSRTYAYQMAKEFSL
EC KdsB 164 RDRFAEGLET----VGDNFLRHLGIYGYRAGFIRRYVNWQPSPL
HI KdsB 164 RDQFMNLQDVQKVQLSDAYLRHIGIYAYRAGFIKQYVQWAPTQL
EC KpsU 163 RN----------AEKARYLKHVGIYAYRRDVLQNYSQLPESMP
CT KdsB 171 FKH----------PTPIYLHIGVYAFRKAFLSEYVKIPPSSL
HD NeuA 206 DIDAIIDLKMAELLLQHEHAII--------------------
HI SiaB 210 DIDSTLDLQLAESLISKEF----------------------
NM SynB 209 DIDTELDLQQAENILNHKES----------------------
EC NeuA 207 DIDDRMDFELAITIQQKKNRQKIDLYQNIHNRINEKRNEFDSVS
SA CpsF 207 DVDTRDDFIHVIGHLFFDYAIREKENKVFYKEGYSRLFNREASK
EC KdsB 204 EHIEMLE---QLRVLWYGEKIHVAVAQEVPGTGVDTPEDLERVR
HI KdsB 208 ENLEKLE---QLRVLYNGERIHVELAKEVPAVGVDTAEDLEKVR
EC KpsU 196 EQAESLE---QLRLMNAGINIR-TFEVAATGPGVDTPACLEKVR
CT KdsB 203 SLAEDLE---QLRVLETGRSIYVHVVQNATGPSVDYPEDITKVE

The N-terminal regions of CMP-NeuAc and CMP-KDO synthetases also share some similarity with other nucleotidyltransferases that catalyze the general reaction:

$$\text{sugar-1-P} + \text{NTP} \text{ ---> } \text{NDP-sugar} + PP_i$$

where NTP is a nucleotide triphosphate. The sequence alignment of several representative sugar nucleotidyltransferases is shown in Fig. 3.11. The conserved domains are aligned with a consensus sequence of ADP-glucose synthetases that is believed to be involved in phosphate binding (Smith and Preiss 1992). This domain, along with a second, were shown to be highly conserved in glucose-1-phosphate nucleotidyltransferases (Thorson et al. 1994). The second domain is not found in CMP-NeuAc, CMP-KDO, or UDP-N-acetylglucosamine synthetases. Based on the conserved positive charges and high sequence similarity in the N-terminus, chemical modifications and site-directed mutagenesis experiments with the *E. coli* enzyme, and similarity between CMP-NeuAc synthetases and other nucleotidyltransferases, it is quite possible that the N-terminal region is involved in binding CTP. There are several other conserved residues and domains within the CMP-NeuAc and CMP-KDO synthetases that are not shared between the classes and may be involved in sugar substrate specificity.

Regarding the role of CMP-NeuAc synthetase in *H. ducreyi*, it has been mentioned in Chapter 1 that many strains contain terminal N-acetyllactosamine (LacNAc) and sialylated LacNAc as part of their LOS (Melaugh et al. 1994; Melaugh et al. 1996). These epitopes are also a component of human blood group antigens and have important roles in molecular and cellular recognition (Schauer 1985; Mandrell et al. 1992). Understanding the function of these sugars in the interaction of *H. ducreyi* with host cells should lead to a better understanding of the pathogenesis of this organism. Recent studies have shown that *H. ducreyi* can adhere to and invade various human cells (Lagergard et al. 1993; Lammel et al. 1993; Brentjens et al. 1994; Totten et al. 1994). As carbohydrates are often involved in cellular recognition (see Chapter 1), LOS may play an important role in this process. In fact, a recent study has shown that an isogenic mutant of *H. ducreyi* with a very truncated LOS structure (lacking LacNAc and sialyl

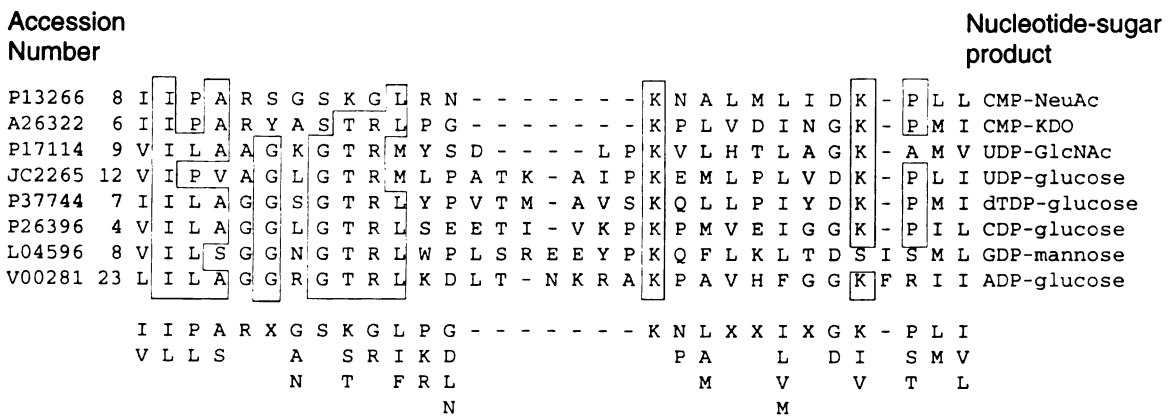


Fig. 3.11. **Sequence alignment of conserved region of some nucleotidyltransferases.** Representative nucleotidyltransferases from *E. coli* and *Salmonella typhimurium* are aligned with a region in ADP-glucose synthetase sequences that is thought to be involved in binding phosphorylated compounds. The residues found in the corresponding position in the nine CMP-NeuAc and CMP-KDO synthetases are indicated below the alignment. An X indicates more than four different residues at that position. The nucleotide sugar product of each enzyme is indicated to the right.

LacNAc) was much less capable of adhering to and invading keratinocytes (Gibson et al. 1997). Sialic acid may offer the bacteria greater protection from the host immune defenses, but exposed terminal galactose may be necessary for the initial infection as has been suggested for *Neisseria gonorrhoeae* LOS (Porat et al. 1995). An isogenic mutant of *H. ducreyi* incapable of producing sialylated LOS will enable a better understanding of the role of sialic acid and the terminal galactose in adhesion to and invasion of host cells and susceptibility of the organism to host defenses. The cloning of the CMP-NeuAc synthetase gene described here has led to the creation of just such a mutant. This mutant and its characterization will be discussed in Chapter 5.

The importance of sialylated molecules in general and as virulence factors in pathogenic bacteria in particular, make understanding the biosynthesis of sialic acid containing molecules an area of great interest. Further study will hopefully better define the role of sialic acid and LOS in the pathogenesis of *H. ducreyi*. With large quantities of recombinant CMP-NeuAc synthetase now available, more detailed characterization of this enzyme can be accomplished, including screening conditions for crystallographic analysis. Along with the information gained from conserved residues and domains in the nine sequences of CMP-NeuAc and CMP-KDO synthetases now available, site-directed mutagenesis and active site labeling with reactive substrate analogs will hopefully lead to a better understanding of the residues necessary for substrate binding and catalysis in CMP-NeuAc synthetases. A greater understanding of this enzyme may be useful not just in the study of *H. ducreyi* pathogenesis, but could eventually lead to effective therapies for pathogens which synthesize sialic acid capsules, such as *E. coli* K1 and *N. meningitidis* strains. These organisms must synthesize CMP-NeuAc to make their capsules and inhibition of the CMP-NeuAc synthetase could severely compromise their virulence.

Towards this goal, active site labeling experiments were conducted to gain more information about important residues on the enzyme. These studies are discussed in the next chapter. The ability to measure the molecular mass of the intact enzyme with ESI-

MS with high accuracy, described in this chapter, would prove to be quite useful in this pursuit.

CHAPTER 4.

Covalent Modification of Lysine-19 and an Essential Arginine in the CTP Binding Site of the *Haemophilus ducreyi* Cytidine 5'-Monophosphate N-acetylneuraminic Acid Synthetase

4.1 INTRODUCTION

Expression of the *H. ducreyi* CMP-NeuAc synthetase in *E. coli* using a T7lac expression vector along with a three step purification procedure resulted in a large quantity of highly pure enzyme (see Chapter 3). This allowed us to conduct detailed studies using several chemical modification reagents to covalently modify and inactivate the CMP-NeuAc synthetase, with the goal of identifying residues important in binding the substrate CTP or involved in catalysis. It has been previously noted that the *E. coli* CMP-NeuAc synthetase is sensitive to lysine and arginine modification reagents, but a detailed kinetic analysis or isolation of labeled peptides was not reported (Vann et al. 1993; Zapata et al. 1995). This chapter describes the chemical modification of the *H. ducreyi* CMP-NeuAc synthetase with lysine and arginine specific modification reagents, the kinetics of inactivation, the isolation of labeled peptides, and their characterization by mass spectrometry.

Periodate oxidized CTP (oCTP), pyridoxal 5'-phosphate (PLP), and phenylglyoxal (PGO) were used to investigate the role of lysine and arginine residues in the CTP binding site of the CMP-NeuAc synthetase from *H. ducreyi.* The reaction of oCTP with the enzyme follows pseudo-first-order saturation kinetics, giving a maximum rate of inactivation of 0.6 min^{-1} and a K_I of 6.0 mM at pH 7.1. Mass spectrometric analysis of the modified enzyme provided data that was consistent with β-elimination of triphosphate after the reaction of oCTP with the enzyme. A fully reduced enzyme-oCTP conjugate, retaining the triphosphate moiety, was obtained by inclusion of $NaBH_3CN$ in the reaction solution.

The β-elimination product of oCTP reacted several times more rapidly with the enzyme compared to equivalent concentrations of oCTP. This compound also formed a stable reduced morpholino adduct with CMP-NeuAc synthetase when the reaction was conducted in the presence of $NaBH_3CN$, and was found to be a useful lysine modifying reagent. The arginine-specific reagent, PGO, reacted to form a 1:1 complex with the enzyme and followed pseudo-first-order kinetics without saturation, indicating a one-step irreversible reaction. The kinetic data was consistent with the modification of as few as one arginine residue per active enzyme unit being responsible for the loss of activity. The substrate CTP was capable of protecting the enzyme to a large degree from inactivation by both oCTP and PGO. PLP reacted less specifically than either oCTP or PGO, labeling multiple residues during inactivation reactions, however CTP was still capable of providing some degree of protection from inactivation. Lysine-19, a residue conserved in CMP-NeuAc synthetases, was identified as being labeled with both the β-elimination product of oCTP and PLP using peptide mapping and mass spectrometry.

4.2 MATERIALS AND METHODS

4.2.1 Materials

N-acetyl arginine, $NaBH_4$, $NaBH_3CN$, $NaIO_4$, pyridoxal 5'-phosphate, phenylglyoxal, Bicine, MOPS, and all nucleotides and periodate oxidized nucleotides were from Sigma. The premixed solution of α-cyano-4-hydroxycinnamic acid was from Hewlett Packard and the solid material was from Aldrich.

4.2.2 Methods

4.2.2.1 *CMP-NeuAc Synthetase*

The expression and purification of the recombinant *H. ducreyi* CMP-NeuAc synthetase was described in Chapter 3. Before use, the enzyme that was stored as an ammonium sulfate suspension was pelleted and residual ammonium sulfate removed by desalting with a Centricon-10 or Centricon-30 concentrator (Amicon). Protein concentration was determined by using an extinction coefficient of 28.2 at 205 nm for a 1 mg/ml solution. The extinction coefficient was calculated using the method described in (Scopes 1987).

4.2.2.2 *Synthesis of 4'5'-Anhydro-2'3'-Dialdehyde Cytidine (Anhydro-oC) and 4'5'-Anhydro-2'3'-Dialdehyde 3,N^4-Etheno-Cytidine (Anhydro-ε-oC)*

The periodate oxidized CTP (2',3'-dialdehyde CTP, oCTP) β-elimination product (anhydro-oC) was prepared by the method described in (Schwartz and Gilham 1972). Briefly, 1.1 ml of 0.2 M CTP (220 μmol) was adjusted to approx. pH 8.5 and 1.1 ml of 0.2 M $NaIO_4$ was added. After 5 min at room temperature the reaction was heated at 45°C for 3 hr. Upon cooling to room temperature, the reaction was loaded on a QAE-Sephadex A-25 column (acetate form) and eluted with water. The UV absorbing material in the flow through fractions was collected and lyophilized. During and after the reaction, the product was protected from light. The concentration was determined by measuring absorbance at 272 nm using an extinction coefficient of 9000 M^{-1} cm^{-1} for cytidine at pH 7. Anhydro-oC has a λ_{max} of 238 nm, which is characteristic of α,β-unsaturated aldehydes (Grant and Lerner 1980). The yield was 34%. The ε-oCTP β-elimination product (anhydro-ε-oC) was prepared in a similar fashion on a smaller scale (12.8 μmol ε-CTP starting material).

An extinction coefficient of 11700 M^{-1} cm^{-1} at 272 nm for ε-cytidine at pH 7 was used to calculate anhydro-ε-oC concentration (Barrio et al. 1972). A broad absorbance maxima was observed at 242 nm. A yield of 50% was obtained.

4.2.2.3 *Inactivation Kinetics*

The rates of inactivation of CMP-NeuAc synthetase with oCTP (A), anhydro-oC (B), and phenylglyoxal (PGO) (C) were determined. (A) The enzyme (0.5 mg/ml) in 0.1 M MOPS, pH 7.1, 25 mM $MgCl_2$ (buffer A) was incubated at 37°C in the presence of 1-25 mM oCTP. A second series of reactions were carried out which also included 25 mM $NaBH_3CN$. Four aliquots (5 µl) were removed at 2.5 or 5 min intervals to measure residual enzyme activity. The aliquots were immediately diluted to 500 µl with 1 mg/ml BSA, 0.2 M NaCl, 1 mM $MgCl_2$, 10 µl of the dilution was added to 90 µl of assay solution and the reaction was incubated at 37°C for 5 min. Quantitation of enzyme activity was done using the anion exchange HPLC method described in Chapter 2. The rate of inactivation (k_{obs}) at each concentration of inactivator was determined from the slope of the plot of $\ln(A/A_0)$ *versus* time, where A is the current enzyme activity and A_0 is the initial enzyme activity. (B) Reactions were carried out as in (A) except the concentration of anhydro-oC varied from 0.5-8 mM and aliquots were removed at 1 or 2.5 min intervals. (C) Reactions with PGO were carried out as in (A) except the concentration of PGO varied from 5-50 mM.

4.2.2.4 *Preparative Labeling and Protection by CTP*

CMP-NeuAc synthetase (0.5 mg/ml) in 250-1000 µl of buffer A was incubated at 37°C in the presence of various concentrations of the following reagents: oCTP, anhydro-oC, anhydro-ε-oC, PLP, and PGO. Some of the reactions with oCTP, anhydro-oC, and anhydro-ε-oC also included 25 mM $NaBH_3CN$. For reactions with aldehyde reagents, the

reactions were quenched after 2.5 to 100 min by chilling on ice, adding 1 volume of ice cold 25 mM $NaBH_4$ in 35 mM NaOH (final pH of solution approx. 7.8), and incubating on ice for 1-2 hrs. PGO reactions were stopped by adding N-acetyl arginine to a final concentration of 50 mM and incubating on ice for 30 min. A second series of reactions were also done in the presence of 5-20 mM CTP. Control reactions without any added inactivator were also completed under the same conditions. The reactions were desalted by passage over a P10 (BioRad) size exclusion column (7.5 x 75 mm, 3.3 ml) or with Centricon-10 concentrators into 50 or 100 mM NH_4HCO_3 pH 8 and stored at -80°C.

4.2.2.5 *Nucleotide Specificity*

CMP-NeuAc synthetase was diluted to 10 μg/ml in 1 mg/ml BSA, 0.2 M NaCl, 1 mM $MgCl_2$ and 10 μl was added to 90 μl of assay mix to give a final concentration of 2 mM NeuAc, 20 mM $MgCl_2$, 0.2 M MOPS, pH 7.1, and 1 mM of nucleotide. The nucleotides tested were CTP, 2'-deoxy-CTP, cytosine β-D-arabinofuranoside triphosphate (ara-CTP), $3,N^4$-etheno-CTP (ε-CTP), UTP, and ATP. The reaction was incubated at 37°C for 5 min and enzyme activity was quantitated using the anion exchange HPLC method described in Chapter 2. Blank reactions were done by adding the BSA solution without CMP-NeuAc synthetase. A second set of reactions was completed using 1 mg/ml CMP-NeuAc synthetase diluted in BSA and incubated at 37°C for 4 hr.

4.2.2.6 *Degradation of Oxidized Nucleotides Under Enzyme Labeling Reaction Conditions*

oCTP (2.5 mM) was incubated at 37°C for 0 or 30 min using the same conditions as for the preparative labeling reactions in a volume of 5 μl. The aldehyde groups were reduced by adding 5 μl of 25 mM $NaBH_4$ in 35 mM NaOH and leaving on ice for 1 hr. The reactions were analyzed using the same anion exchange method described in Chapter 2

with a minor modification to the gradient. The column was loaded in 100% H_2O without any NH_4HCO_2 to avoid overlap of the β-elimination product peak (which does not bind to the column) and any nucleotide monophosphate impurities present in the sample which elute close by under the usual gradient conditions. The percentage of β-elimination product was taken as the ratio of the area of the unretained peak to the total combined area of all nucleotide peaks (due to the β-elimination product, mono-, di-, and triphosphate species). oCTP, oUTP, and oATP (1 mM) were also analyzed for the amounts of β-elimination product present in the preparations under the same conditions as above but without $NaBH_3CN$ or CMP-NeuAc synthetase included. To assess the effect of pH on the extent of the β-elimination reaction, some reactions were carried out with 0.1 M Bicine pH 9.0 replacing the MOPS pH 7.1 buffer. Reactions in Bicine buffer were reduced as above except the 25 mM $NaBH_4$ solution contained only 1 mM NaOH to avoid substantially raising the pH. Some samples included 0.2 M $(NH_4)_2SO_4$ to determine the effect of ammonia on the β-elimination reaction.

4.2.2.7 *Tryptic Peptide Mapping*

In order to identify specific sites of chemical modification of the CMP-NeuAc synthetase, enzyme (approx. 50-100 μg in 500 μl 100 mM NH_4HCO_3) from the preparative labeling reactions was digested with 3 μg of trypsin (in 50 μl of 50 mM AcOH) (Promega, sequencing grade) at 37°C for 2 hrs. A second identical aliquot of trypsin was added and the digestions continued for another 2 hrs. The digests were stored at -80°C until purification by reverse phase chromatography on a Vydac C18 column (2.1 x 250 mm) at a flow rate of 0.2 ml/min with the following gradient: 7.1% B for 5 min, 7.1-85.7% B in 55 min, 85.7-100% B in 2 min, and 100% B for 5 min. Solvent A was 0.1% triflouroacetic acid (TFA) in H_2O and solvent B was 70% CH_3CN containing 0.08% TFA. Absorbance was monitored at 280 nm for peptides modified by anhydro-oC or oCTP, 288 nm for peptides modified by anhydro-ε-oC, and 324 nm for peptides modified by PLP

using an Applied Biosystems 1000S diode array detector. Peaks were collected for mass spectrometric analysis and stored at -80°C until needed. Two of the three labeled peptides that were to be sequenced by Edman degradation required further purification before sequencing. The peptides were rechromatographed on the same C18 column with a gradient of CH_3CN using 0.1% HCO_2H instead of TFA.

4.2.2.8 *Mass Spectrometry of Proteins and Tryptic Peptides*

Chemically modified protein and peptide samples were subjected to electrospray ionization (ESI) and matrix assisted laser desorption ionization (MALDI) mass spectrometry to determine the type of covalent modification that had occurred and the extent of reaction. Protein samples were largely analyzed by ESI-MS, and typically 1-5 µl of desalted enzyme (approx. 5-15 µg) was injected into a stream of H_2O/CH_3CN (1:1) (with 1% acetic acid or formic acid) at a flow rate of 10-25 µl/min which was coupled to the ESI source of a Fison Platform quadrupole mass spectrometer (Fisons Instruments, Manchester U.K.) or an API 300 triple quadrupole mass spectrometer (Perkin-Elmer Sciex) run in the positive-ion mode. Horse heart myoglobin (average M_r = 16951.5) was used as a reference compound and external calibrant and a typical mass accuracy of ± 0.01% was obtained. The molecular mass was calculated using the maximum entropy algorithim from the computer program MassLynx 2.1 (Fisons Instruments) with settings of 0.5 or 1.0 Da/channel resolution and 0.5 or 1.0 Da peak width at half height. For MALDI-MS analysis of proteins, a PerSeptive Biosystems (Framingham, MA) Voyager time-of-flight mass spectrometer was operated in the positive-ion mode. Typically enzyme (0.1 to 1 mg/ml) that was desalted in H_2O or 5-20 mM NH_4HCO_3 was mixed 1:1 with α-cyano-4-hydroxycinnamic acid (saturated solution in 70% CH_3CN 0.08% TFA) and 1 µl spotted on the sample plate.

For ESI-MS analysis of peptides, 1-5 µl aliquots of HPLC fractions were injected under the same conditions as the protein samples. Polypropylene glycol was used as an

external calibrant. Molecular mass was calculated as for the protein samples. For MALDI-MS of peptides, PerSeptive Biosystems Voyager-DE and Voyager Elite delayed extraction time-of-flight mass spectrometers were used (Vestal et al. 1995). Peptide fractions from proteolytic digests were mixed 1:1 with a solution of α-cyano-4-hydroxycinnamic acid from Hewlett Packard and 1 μl spotted on to the sample plate. The instruments were operated in the positive-ion mode. Calibration was performed using angiotensin II (monoisotopic MH^+ = 1046.54 Da) and α-MSH (monoisotopic MH^+ = 1664.80 Da) as external standards. When HPLC fractions with labeled peptides also contained other, unmodified tryptic peptides of known mass, these were used for internal calibration to obtain greater mass accuracy. For post source decay (PSD) analysis (Kaufmann et al. 1993), peptides were also mixed 1:1 with the α-cyano-4-hydroxycinnamic acid solution, 1 μl spotted on the sample slide, and analyzed with a VG TofSpec SE mass spectrometer (Fisons Instruments) with the course laser power set to the maximum. The instrument was calibrated in PSD mode using the fragment ions generated from the standard peptide, ACTH 18-39.

4.2.2.9 *Amino Acid Sequencing*

Peptide sequencing was performed by the UCSF Biomolecular Resource Center using an Applied Biosystems Procise Protein Sequencing System (Applied Biosystems/Perkin-Elmer).

4.3 RESULTS

Inactivation by Phenylglyoxal (PGO). To assess the role of arginine residues in CMP-NeuAc synthetase, the enzyme was incubated with PGO, a chemical modification reagent that is highly selective for arginine residues in proteins (Takahashi 1968; Powers and Riordan 1975; Takahashi 1977). PGO inactivated the enzyme in a time

and concentration dependent manner, following pseudo-first-order kinetics. The plot of observed rate constants *versus* PGO concentration yields a straight line that passes very close to the origin (Fig. 4.1A), indicating that the modification occurs by a simple irreversible reaction without a prior binding step (Cardemil 1987). The reaction can be described by:

$$E + PGO \xrightarrow{k_1} E\text{-}PGO \qquad (1)$$

where E represents free enzyme and E-PGO the inactive enzyme-PGO covalent complex. When [PGO] >> [E], the observed inactivation rate is simply, $k_{obs} = k_1[PGO]$. The reaction order can be determined for this mechanism from the slope of the plot of log k_{obs} *versus* log [PGO] (Cardemil 1987). The slope was calculated as 1.3 (Fig. 4.1B), indicating that the modification of as few as one arginine residue in CMP-NeuAc synthetase is responsible for the loss of enzyme activity.

Inactivation by oCTP and Other Aldehyde Reagents. Lysine residues in CMP-NeuAc synthetase were targeted with oCTP and other aldehyde reagents. Saturation kinetics were observed for the reaction of oCTP with CMP-NeuAc synthetase, with or without $NaBH_3CN$ present in the reaction (Fig. 4.2A), suggesting that a reversible complex is formed prior to inactivation (Cardemil 1987). The mechanism is described by:

$$E + oCTP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E * oCTP \xrightarrow{k_{max}} E\text{-}oCTP \qquad (2)$$

where E represents free enzyme, E * oCTP is the reversible, non-covalent complex of enzyme and oCTP, and E-oCTP the inactive, covalent complex (Cardemil 1987). The observed inactivation rate is:

$$k_{obs} = k_{max}[oCTP]/(K_I + [oCTP]) \qquad (3)$$

where k_{max} is the maximum rate of inactivation at saturating concentrations of oCTP. K_I is the concentration of oCTP that yields a k_{obs} that is one half of k_{max} and is given as:

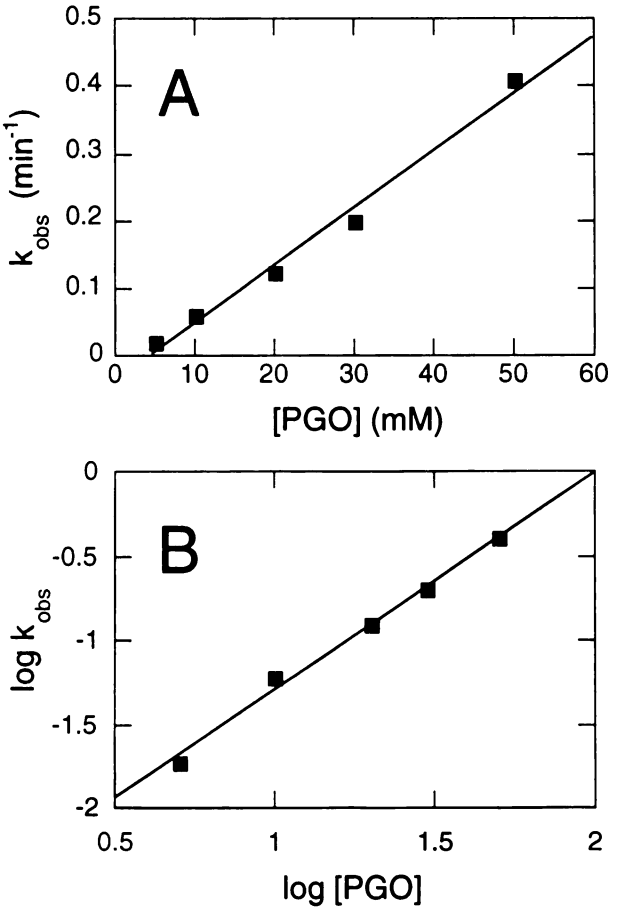


Fig. 4.1. **Concentration dependence of the rate of inactivation (k_{obs}) of CMP-NeuAc synthetase by phenylglyoxal.** (A) CMP-NeuAc synthetase was incubated with varying concentrations of PGO at pH 7.1 and 37°C (see "Methods" for details). Aliquots were removed and assayed for residual enzyme activity over the course of the reaction. The rate of inactivation (k_{obs}) for each PGO concentration was determined from the slope of the semilog plot of residual activity *versus* time. A second order rate constant of 8.5 M^{-1} min^{-1} was calculated from the slope of the plot of k_{obs} versus PGO concentration. (B) Logarithmic plot of the data shown in (A). The slope of the double log plot is 1.3.

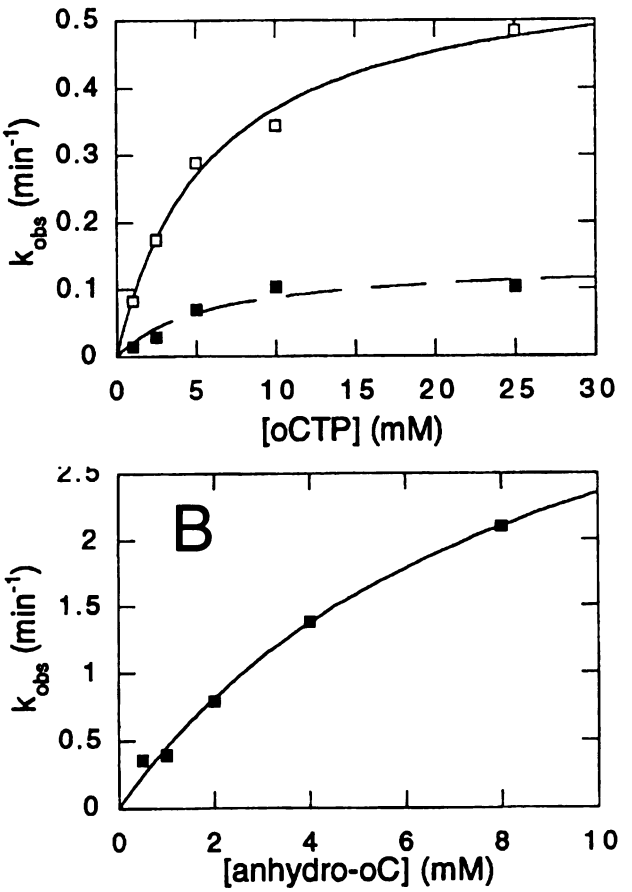


Fig. 4.2. **Concentration dependence of the rate of inactivation (k_{obs}) of CMP-NeuAc synthetase by oCTP and anhydro-oC.** CMP-NeuAc synthetase was incubated with varying concentrations of oCTP (A) or anhydro-oC (B) at pH 7.1 and 37°C (see "Methods" for details). The inactivation rates (k_{obs}) were determined as described in Fig. 4.1. The data was fit to the equation $k_{obs} = k_{max}[I]/(K_I + [I])$, where [I] is the concentration of oCTP or anhydro-oC, with the computer program KaleidaGraph (Synergy Software; Reading, PA). (A) The reactions were done in the absence of $NaBH_3CN$ (open squares) or with 25 mM $NaBH_3CN$ (filled squares) present. A k_{max} of 0.14 ± 0.02 min^{-1} and 0.59 ± 0.04 min^{-1} was obtained for the reaction with and without $NaBH_3CN$, respectively. A K_I of 5.8 ± 2.7 mM and 6.0 ± 0.9 mM was obtained for the reaction with and without $NaBH_3CN$, respectively (all values reported as plus or minus the standard error). (B) A k_{max} of 4.5 ± 0.7 min^{-1} and a K_I of 9.1 ± 2.3 mM were calculated for the reaction of CMP-NeuAc synthetase with anhydro-oC.

$$K_I = (k_{-1} + k_{max})/k_1 \quad (4)$$

The maximum rate of inactivation in the presence of $NaBH_3CN$ was approximately 4 times slower than the reaction without $NaBH_3CN$ (0.14 min^{-1} versus 0.59 min^{-1}). The K_I of the two reactions were very similar (5.8 and 6.0 mM).

The reaction of anhydro-oC with the enzyme followed pseudo-first-order kinetics and it appeared that saturation kinetics were followed as well (Fig. 4.2B). However, measurement of inactivation rates at concentrations of anhydro-oC greater than 8 mM was not possible because of the speed of the reaction (< 1% residual activity in 2 min with 8 mM anhydro-oC). The inactivation rate of 5 mM anhydro-oC is approximately 6 times faster than the rate of inactivation from 5 mM oCTP. Anhydro-ε-oC reacted at a similar rate to anhydro-oC. The presence of $NaBH_3CN$ in the anhydro-oC reaction slowed down the inactivation rate as was observed with oCTP (data not shown).

Because oCTP and anhydro-oC were more specific inactivators than PLP, the kinetics of inactivation of PLP were not analyzed in as much detail. However, a pattern of fast inactivation followed by a much slower phase of inactivation was observed at the several different concentrations of PLP tested. Biphasic kinetics are commonly observed for enzyme inactivations by PLP (Lilley and Engel 1992; Bazaes et al. 1993). In an attempt to minimize nonspecific modification, high concentrations of PLP were incubated with CMP-NeuAc synthetase for very short times before quenching the reactions. When low concentrations of PLP were used for long incubation periods, the enzyme would become covalently modified, but without losing very much enzyme activity.

In addition to oCTP, both oUTP and oATP were capable of inactivating CMP-NeuAc synthetase. The enzyme was inactivated to a similar extent with all three of the oxidized nucleotide triphosphates tested (Table 4.1).

Protection by CTP. The substrate CTP was able to protect CMP-NeuAc synthetase, to a greater or lesser degree, from inactivation by the lysine and arginine modification reagents described above (Table 4.1). Neither 2'-deoxy-CTP or UTP were able to protect the enzyme from inactivation (data not shown).

Table 4.1. Inactivation of CMP-NeuAc synthetase and protection by CTP

Reaction conditions [a]	time	residual activity [b]	residual activity with CTP [c]
	(min)	*(%)*	*(%)*
2.5 mM oCTP	30	2	nd
2.5 mM oUTP	30	3	nd
2.5 mM oATP	30	4	nd
2.5 mM oCTP, 25 mM $NaBH_3CN$	30	64	nd
0.5 mM anhydro-oC, 25 mM $NaBH_3CN$	30	49	nd
10 mM PGO	30	15	90 (20 mM)
5 mM PLP	2.5	77	91 (10 mM)
1 mM oCTP, 25 mM $NaBH_3CN$	60	59	99 (5 mM)
0.1 mM anhydro-oC, 25 mM $NaBH_3CN$	100	66	89 (5 mM)
0.1 mM anhydro-ε-oC, 25 mM $NaBH_3CN$	100	50	82 (5 mM)

[a] Reactions contained 0.5 mg/ml CMP-NeuAc synthetase and were incubated at 37°C for the indicated period of time in 0.1 M MOPS pH 7.1, 25 mM $MgCl_2$. When samples were intended for ESI-MS analysis or isolation of labeled peptides, reaction conditions were chosen to limit the degree of inactivation and minimize non-specific labeling.

[b] Activity is expressed relative to the initial activity of the sample at t = 0 min. When no inactivator was present, CMP-NeuAc synthetase was stable under these conditions, and control reactions showed no loss of activity.

[c] The amount of CTP included is indicated in parenthesis. (nd, not determined)

Stability of oCTP Under Enzyme Labeling Conditions. Periodate oxidized nucleotides are not very stable compounds and can readily undergo β-elimination reactions under fairly mild conditions. Because anhydro-oC inactivated CMP-NeuAc synthetase several times faster than oCTP, it was important to determine the amount of anhydro-oC present initially in the oCTP preparation and its generation over the course of a reaction under the conditions used. High levels of anhydro-oC impurity in the oCTP sample would complicate interpretation of kinetic and mass spectrometric analysis of oCTP reactions. oCTP was fairly stable at 37°C in 0.1 M MOPS, pH 7.1. In 30 min, the amount of anhydro-oC present only increased to 5.5% from the initial amount of approx. 3% (Table 4.2). The presence of CMP-NeuAc synthetase in the buffer did not affect the results. However, when ammonia was present (as ammonium sulfate), the degradation was greatly accelerated. The opposite effect was observed for the reaction at pH 9.0 in Bicine buffer. In the absence of ammonia, the amount of anhydro-oC increased to 23% in 30 min, while the addition of 0.2 M $(NH_4)_2SO_4$ lessened the amount formed to 12.4%. The addition compounds of primary amines and ammonia with periodate oxidized nucleotides are known to readily lose phosphate at pH values less than 8, yet are fairly stable at pH 9.5 (Khym and Cohn 1961; Khym 1963). The effects of pH and ammonia on the degradation of oCTP are in agreement with those findings.

Nucleotide Specificity. Because periodate oxidized nucleotides other than oCTP and anhydro-oC inactivated CMP-NeuAc synthetase, we attempted to better define the nucleotide substrate requirements of the enzyme. The enzyme appears to be quite specific for CTP as its nucleotide substrate. Although it can tolerate the changes at the 2' position in 2'-deoxy-CTP and ara-CTP, the activity is only 1-3% of the activity with CTP (Table 4.3). Nucleotides with differences in the base (UTP, ε-CTP, and ATP) were not used as substrates. At first, ε-CTP appeared to be used as a substrate when the reaction was only allowed to proceed in the linear initial velocity range. However, when a much greater amount of enzyme (1 mg/ml *versus* 10 μg/ml) was used along with longer reaction times (4 hr *versus* 5 min), the size of the product peak barely increased (data not shown).

Table 4.2. Degradation of oxidized nucleotides under enzyme labeling conditions

Reaction conditions [a]	β-elimination product [b] (%)	
	0 min	30 min
MOPS pH 7.1	2.9 (0.2)	5.5 (0.2)
MOPS/0.2 $M(NH_4)_2SO_4$	nd	33.9 (2.2)
MOPS/0.5 mg/ml CMP-NeuAc synthetase	nd	5.6 (0.3)
MOPS/0.5 mg/ml CMP-NeuAc synthetase/25 mM $NaBH_3CN$	nd	5.5 (0.1)
Bicine pH 9.0	3.2 (0.1)	22.9 (0.4)
Bicine/0.2 $M(NH_4)_2SO_4$	nd	12.4 (0.3)
Bicine/0.5 mg/ml CMP-NeuAc synthetase	nd	18.5 (3.4)
Bicine/0.5 mg/ml CMP-NeuAc synthetase/25 mM $NaBH_3CN$	nd	10.9 (0.5)
MOPS (1 mM oCTP)	3.4	5.6
MOPS (1 mM oUTP)	13.0	15.3
MOPS (1 mM oATP)	6.4	9.0

[a] Reactions contained 2.5 mM oCTP unless otherwise indicated. The MOPS and Bicine buffers also contained 25 mM $MgCl_2$. The samples were incubated at 37°C for 0 or 30 min before reduction with $NaBH_4$ and HPLC analysis (see "Methods" for details).

[b] The values reported are the mean of three experiments with the standard deviation in parenthesis, except for the last three reactions. (nd, not determined)

Table 4.3. Nucleotide substrate specificity of CMP-NeuAc synthetase

Nucleotide	Relative activity [a] (%)
CTP	100.0 (2.2)
2'-deoxy-CTP	3.3 (0.1)
ara-CTP	0.9 (0.1)
ε-CTP	3.4 (0.1)
UTP	0.0 (0.04)
ATP	0.0 (0.1)

[a] The activity is expressed relative to the reaction with CTP in which 17% of CTP (the limiting substrate) was consumed. The reactions included 10 μg/ml CMP-NeuAc synthetase, 2 mM NeuAc, 20 mM $MgCl_2$, 0.2 M MOPS, pH 7.1, and 1 mM of nucleotide in a volume of 100 μl and were incubated at 37°C for 5 min. A blank reaction that did not include enzyme was subtracted form the nucleotide reactions. The values reported are the mean of three experiments with the standard deviation in parenthesis.

In contrast, under these conditions, 2'-deoxy-CTP and ara-CTP were converted to the nucleotide sialic acid product to greater than 30% and 50% respectively. It seems likely that the small amount of product formed (approx. 1%) is CMP-NeuAc arising from residual CTP present in the ε-CTP preparation. Indeed, electrospray mass spectrometric analysis of the ε-CTP preparation in negative-ion mode revealed an ion of low abundance at 482 Da, consistent with the deprotonated molecular ion of CTP (mass = 483 Da).

Characterization of Covalent Enzyme Modifications. ESI-MS of chemically modified enzyme preparations was used to determine the type of covalent modification that had occurred and to obtain an estimate of the extent of the labeling reaction. When oCTP was incubated with CMP-NeuAc synthetase without $NaBH_3CN$ present, a species approx. 203 Da greater in mass than the unmodified enzyme was observed (Fig. 4.3A). This mass increase is consistent with the covalent addition of oCTP to the enzyme with β-elimination of the triphosphate moiety as shown in Fig. 4.4 (2B, 3B or 5B). Structures 1B and 4B can be eliminated as possibilities because the mass added by these covalent modifications is well outside of the experimental error of the technique. In the presence of $NaBH_3CN$, the reaction of oCTP with the enzyme produced a modification approx. 450 Da greater in mass than the unmodified enzyme, consistent with structure 4A in Fig. 4.4, along with a smaller amount of the product that had lost triphosphate (Fig. 4.3B). This fully reduced morpholino adduct is analogous to a model compound prepared from the reaction of oAdo and glycine in the presence of $NaBH_3CN$ (Rayford et al. 1985). A similar result was obtained for the reaction of anhydro-oC with the enzyme in the presence of $NaBH_3CN$. The reaction produced a species with a mass corresponding to covalent modification by anhydro-oC as the reduced morpholino derivative (4B in Fig. 4.4), as well (Fig. 4.3C).

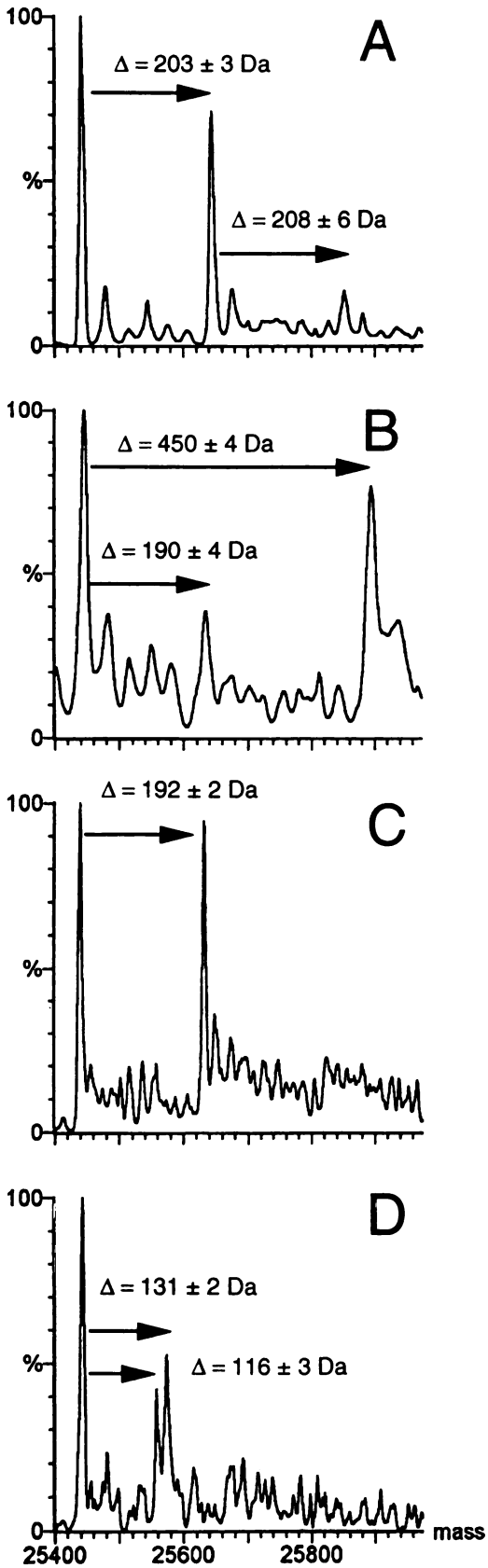
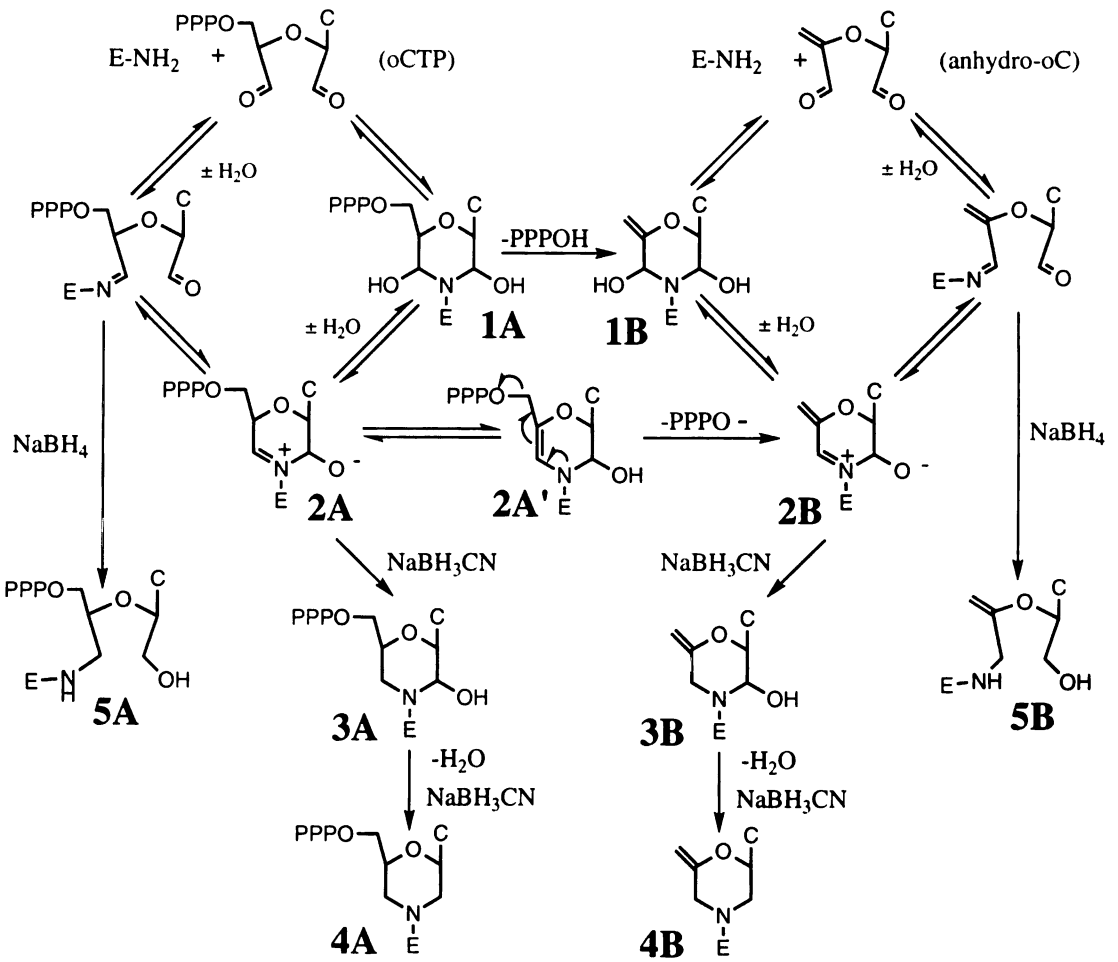


Fig. 4.3. **Electrospray mass spectra of covalently modified CMP-NeuAc synthetase.** CMP-NeuAc synthetase that had undergone reaction with chemical modification reagents was desalted and then analyzed by ESI-MS. The raw data was baseline subtracted and transformed using a maximum entropy algorithim from the MassLynx computer program to produce the spectra shown. The arrows indicate the shift in mass between the unmodified enzyme (25440 Da) and the covalently modified species. CMP-NeuAc synthetase had been modified under the following conditions: (A) 2.5 mM oCTP for 30 min, (B) 2.5 mM oCTP, 25 mM $NaBH_3CN$ for 30 min, (C) 0.5 mM anhydro-oC, 25 mM $NaBH_3CN$ for 30 min, or (D) 10 mM PGO for 30 min. All reactions were done in 0.1 M MOPS pH 7.1, 25 mM $MgCl_2$ at 37°C.

Fig. 4.4. **Possible reactions of oCTP and anhydro-oC with primary amines.** The dialdehyde reagents, oCTP and anhydro-oC, can react with primary amines to form Schiff bases which can be reduced to the stable secondary amines 5A and 5B. oCTP and anhydro-oC are shown as free dialdehydes in the figure, however, they are at equilibrium with hydrated species. Lowe and Beechey found that very little free aldehyde is present at equilibrium in preparations of oATP (although it reacts as an aldehyde), and it exists as a mixture of cyclic hemiacetals and the dihydrate (Lowe and Beechey 1982). The adenosine analog of anhydro-oC contains a free aldehyde at the 3' position due to conjugation with the double bond, while the 2' aldehyde exists as the hydrate (Lowe and Beechey 1982). Alternatively, these compounds can react to form the dihydroxymorpholino structures 1A and 1B. In the presence of $NaBH_3CN$ 1A and 1B can be reduced, after undergoing a dehydration reaction to form 2A and 2B, to the more stable 4A and 4B, respectively. In many cases, when $NaBH_3CN$ is not present, the reaction product of a dialdehyde nucleotide with an enzyme has been found to have lost phosphate. The β-elimination of triphosphate, going from 1A to 1B, can occur under basic conditions, but does not explain why the reaction should occur any faster on the enzyme than in solution. The dehydration of 1A to 2A is an acid catalyzed reaction, which agrees very well with the observation that products of the reaction of primary amines and dialdehyde nucleotides lose phosphate quite slowly at pH > 8 (Khym 1963). The most likely route of formation of 2B is by dehydration of 1A to form 2A, which is in equilibrium with the enamine 2A'. The enamine is able to rapidly react to eliminate phosphate as noted by Lowe and Beechey (Lowe and Beechey 1982) to yield 2B. The mass differences for the covalent modifications with respect to the intact enzyme are as follows: 1A, 481 Da; 2A, 463 Da; 3A, 465 Da; 4A, 449 Da; 5A, 467 Da; 1B, 223 Da; 2B, 205 Da; 3B, 207 Da; 4B, 191 Da; 5B, 209 Da.


E-NH2 +
(oCTP)
E-NH2 +
(anhydro-oC)
± H2O
-PPPOH
1A
1B
2A
2A'
2B
-PPPO -
NaBH4
NaBH3CN
5A
3A
3B
5B
-H2O
4A
4B

PGO can react with arginine residues in proteins to form 2:1 or 1:1 complexes, which can possibly undergo rearrangement reactions (Takahashi 1968; Borders and Riordan 1975; Vandenbunder et al. 1981; Bjerrum 1989). Two modified enzyme species were observed for the reaction of PGO with CMP-NeuAc synthetase (Fig. 4.3D). Mass differences of approx. 131 Da and 116 Da are most consistent with formation of a 1:1 complex of PGO with the enzyme and loss of H_2O from the product (Fig. 4.5).

The reaction of PLP with CMP-NeuAc synthetase produced multiple enzyme species with masses separated by an average of 231 Da (range: 229-234 Da) (Fig. 4.6). The data is consistent with from 1 to 7 PLP molecules covalently attached and reduced on to the enzyme with an average of 4.0 (calculated from the area of the peaks). The reaction of PLP with the enzyme in the presence of CTP resulted in a somewhat less modified product, with an average of 3.1 PLP molecules per enzyme (data not shown).

Peptide Mapping and Identification of Labeled Peptides. To identify the specific amino acids modified by the various inactivating reagents, CMP-NeuAc synthetase that had been covalently modified, in the presence or absence of CTP, was desalted, digested with trypsin, and the peptides separated by reverse phase HPLC. Many peptides covalently modified with PLP were observed in digests of enzyme labeled with PLP in the presence or absence of CTP. However, two peaks were present in the unprotected sample that were much smaller in the CTP protected sample (Fig. 4.7). The two fractions were analyzed by MALDI-MS and ESI-MS and based on their observed molecular masses, tentatively identified as extended tryptic peptides that were covalently modified with one or two PLP molecules. Analysis of peak 1 by ESI-MS revealed a peptide with an average mass of 2672.6 Da, consistent with the tryptic peptide 11-32 (see Table 4.4) modified with 2 PLP molecules. The peptide sequence and location of the modifications were determined by partial Edman sequencing which yielded the following sequence: AGS(X)GIKD(X)N, where (X) denotes that no PTH amino acid derivative was identified. The first cycle produced 12 pmol of alanine which dropped to 2 pmol of asparagine by the tenth cycle, at which time the sequencing was stopped. The PTH derivative of PLP-lysine was not

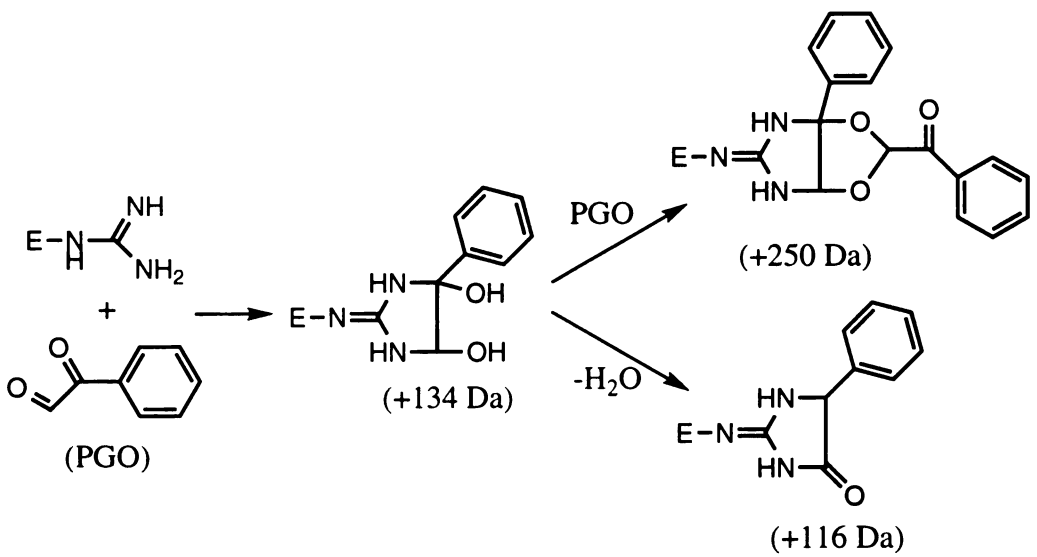


Fig. 4.5. **Possible reactions of PGO with arginine.** PGO can react with arginine residues to form 1:1 or 2:1 complexes. The difference in mass due to the covalent modification with respect to the intact enzyme is listed in parenthesis.

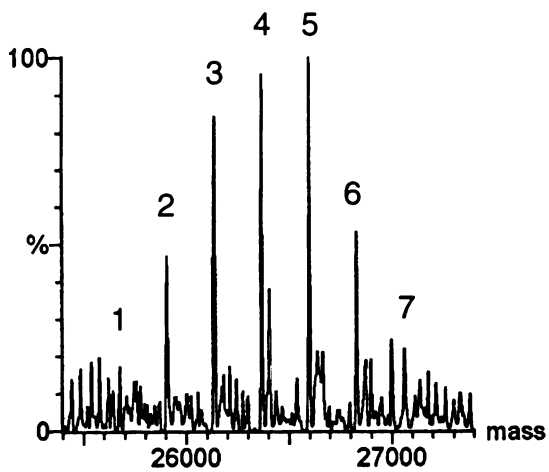


Fig. 4.6. **Electrospray mass spectra of CMP-NeuAc synthetase modified by PLP.** The CMP-NeuAc synthetase sample modified by PLP and the raw ESI-MS data were treated in the same manner as described in Fig. 4.3. The modified enzyme species observed differ in mass by 231 Da, the mass difference expected for covalent modification by PLP. PLP reacted much more extensively with CMP-NeuAc synthetase than any of the other inactivators, with as many as seven PLP molecules incorporated per enzyme molecule and little or no unmodified enzyme remaining. The numbers above the peaks correspond to the number of PLP molecules covalently attached to the enzyme.

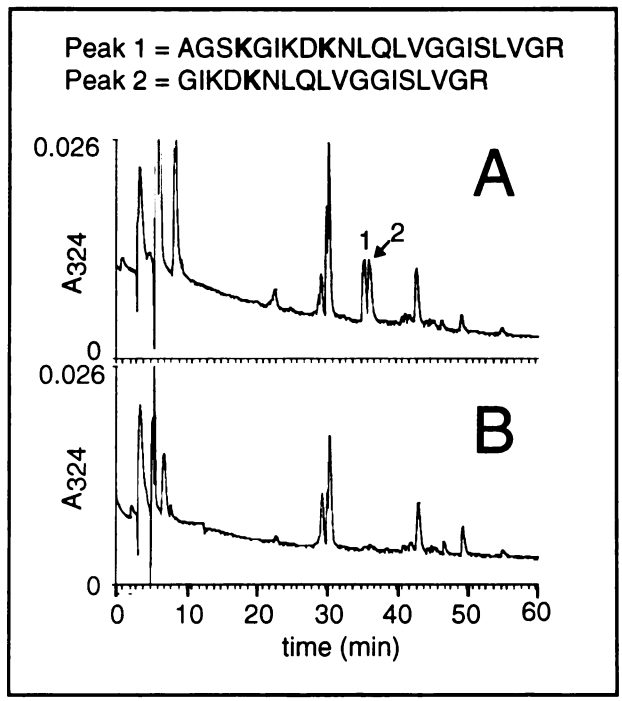


Fig. 4.7. **HPLC separation of the tryptic digest of CMP-NeuAc synthetase covalently modified by PLP.** CMP-NeuAc synthetase was covalently modified by 5 mM PLP in the absence (A) or presence (B) of 10 mM CTP for 2.5 min at 37°C in 0.1 M MOPS pH 7.1, 25 mM $MgCl_2$. After reduction by $NaBH_4$ and desalting (see "Methods" for details), equivalent amounts of the modified samples were digested with trypsin for 4 hrs at 37°C and the peptides separated on a Vydac C18 column (2.1 x 250 mm). The column was eluted with a gradient from 5-70% CH_3CN in H_2O containing TFA as an ion pair reagent. Absorbance was monitored at 324 nm, a wavelength at which PLP modified peptides strongly absorb with virtually no absorbance from unmodified peptides. Two peaks (labeled 1 and 2) were identified in the digest of enzyme modified by PLP in the absence of CTP (A) that were almost completely absent in the digest of enzyme modified in the presence of CTP (B). The peptides identified in peaks 1 and 2 are listed above the chromatograms and covalently modified residues are shown in bold.

Table 4.4. Covalently modified tryptic peptides

HPLC fraction	peptide[a]	calc. mass[b]	peptide mass[b]	Δ mass	residues[c]
		(Da)	*(Da)*	*(Da)*	
PLP peak 1[d]	11-32	2672.6	2210.6	462.0	K_{14}, K_{19}
PLP peak 2[d]	15-32	2098.0	1867.2	230.8	K_{19}
	15-32	2329.0	1867.2	461.8	K_{17}, K_{19}
anhydro-ε-oC peak 1[e]	3-10	1095.8	880.6	215.2	K_3
anhydro-ε-oC peak 2[e]	18-32	1783.1	1567.9	215.2	K_{19}
		1785.1		217.2	
anhydro-oC peak 1	3-10	1071.7	880.6	191.1	K_3
anhydro-oC peak 2	18-32	1759.0	1567.9	191.1	K_{19}
		1761.0		193.1	

[a] The numbers refer to the primary sequence of the *H. ducreyi* CMP-NeuAc synthetase (3-10: **K**IAIIPAR, 11-32: AGS**K**GI**K**D**K**NLQLVGGISLVGR, 15-32: GI**K**D**K**NLQLVGGISLVGR, 18-32: D**K**NLQLVGGISLVGR). Lysine residues, the target of these aldehyde reagents, are shown in bold.

[b] The calculated mass and peptide mass are given as average masses for the three peptides from PLP peak 1 and PLP peak 2. Their calculated masses were determined by ESI-MS. The masses are monoisotopic for all the other peptides and their calculated masses were determined by MALDI-MS.

[c] The position of the covalent modification was determined by Edman degradation for the PLP modified peptides and anhydro-oC peak 2. The position of the label in the anhydro-ε-oC peak 2 is assumed by its mass and analogy with anhydro-oC peak 2. The position of the modification in the anhydro-oC peak 1 and anhydro-ε-oC peak 1 peptides was determined by PSD analysis.

[d] From Fig. 4.7.

[e] From Fig. 4.8.

expected to yield a peak under the normal elution conditions (Lilley and Engel 1992). The middle lysine in the sequence was clearly observed in cycle seven with no evidence of a PTH-lysine peak in cycle 4 or 9. These blank cycles, along with the known enzyme primary sequence, the 324 nm absorbance, and the peptide mass, are strong evidence for modification of K_{14} and K_{19} by PLP. Peak 2 contained a peptide with an average mass of 2098.0 Da which is consistent with the tryptic peptide 15-32 modified with 1 PLP molecule. Six cycles of Edman sequencing gave the following sequence: GIKD(X)N, with greater than 30 pmol of glycine in the first cycle and 25 pmol of asparagine in the last cycle. A yield of 25 pmol of lysine was obtained in cycle 3 with no evidence of PTH-lysine in cycle 5, indicating that K_{19} was labeled in this peptide as well. Peak 2 also contained a smaller amount of a 2329.0 Da species presumably corresponding to the same peptide with both lysines labeled. Interestingly, the PLP modifications were not stable to MALDI-MS analysis. The predominant ion observed for all PLP labeled peptides was always the unmodified peptide with lower intensity ions at +231 Da (addition of PLP) and +134-135 Da (addition of PLP with loss of H_2PO_4). In reflectron mode, the +231 Da peak was not observed at all.

Both oCTP and anhydro-oC were more effective at inactivating the enzyme than PLP at equivalent concentrations and enzyme activity was protected to a greater degree with CTP using these reagents. Because of difficulties in isolating peptides labeled with oCTP, anhydro-oC and the etheno derivative, anhydro-ε-oC, were used to obtain labeled peptides. Digests of enzyme labeled with anhydro-ε-oC in the presence or absence of CTP are shown in Fig. 4.8. Because of the characteristic absorbance of ε-cytidine which extends out to 300 nm, labeled peptides were readily identified from their UV spectra obtained with the diode array HPLC detector. Two labeled peptides were detected, one of which was only present in the digest of the unprotected enzyme (peak 2 in Fig. 4.8). Very similar HPLC chromatograms were obtained for the two analogous digests of anhydro-oC labeled enzyme (data not shown). Peak 1 contained a peptide with a monoisotopic mass of 1095.8 Da (Table 4.4). This corresponds to the tryptic peptide 3-10 covalently modified with

anhydro-ε-oC as a reduced morpholino derivative (4B in Fig. 4.4). The analogous peptide from the anhydro-oC digest had a monoisotopic mass of 1071.7 Da, 24 Da lower, which is the mass difference between ε-cytidine and cytidine. Under PSD conditions almost identical spectra were obtained for the two peptides, yielding an extensive series of y fragment ions (y_1, y_3, y_4, y_5, y_7; 175.0, 343.7, 457.2, 569.9, 754.1 Da), along with several internal fragment ions, and an abundant fragment ion corresponding to loss of the cytidine or ε-cytidine base from the intact labeled peptide. This confirmed the peptide sequence and position of the label for both of these peptides as K'IAIIPAR, where K' indicates a labeled lysine residue. However, both of these peptide masses were also observed in the digests of enzyme protected by CTP and can be attributed to nonspecific labeling. Peak 2, only observed in the digest of unprotected enzyme, contained a peptide with a monoisotopic mass of 1783.1 Da by MALDI-MS while the analogous peptide labeled with anhydro-oC had a monoisotopic mass of 1759.1 Da, again 24 Da lower. These are consistent with the tryptic peptide 18-32 labeled with anhydro-ε-oC or anhydro-oC as the fully reduced morpholino adduct (4B in Fig. 4.4). Both fractions also contained a mass that was 2 Da higher. The +2 Da species was also observed in ESI-MS analysis of both fractions. Presumably this 2 Da greater mass is due to reduction of the double bond as well as the quaternary ammonium by 1,4 addition of cyanoborohydride, similar to the reduction of conjugated carbonyls by cyanoborohydride (Hutchins and Kandasamy 1975). It is not clear why a +2 Da species was not also observed for the labeled peptides from peak 1. MALDI-PSD analysis did not provide enough sequence ions to confirm the peptide sequence. However, as with the peptides from peak 1, the major fragment ion formed under MALDI-PSD was due to a fragmentation that corresponds to loss of the cytidine (-110 Da from protonated MH^+ ion) or ε-cytidine (-134 Da from protonated MH^+ ion) base, indicating that these peptides are indeed labeled (data not shown). Partial Edman sequencing of peak 2 from the anhydro-oC labeled enzyme gave the following sequence: D(X)(-)LQ, with 2.9 pmol aspartic acid in the first cycle and 1.2

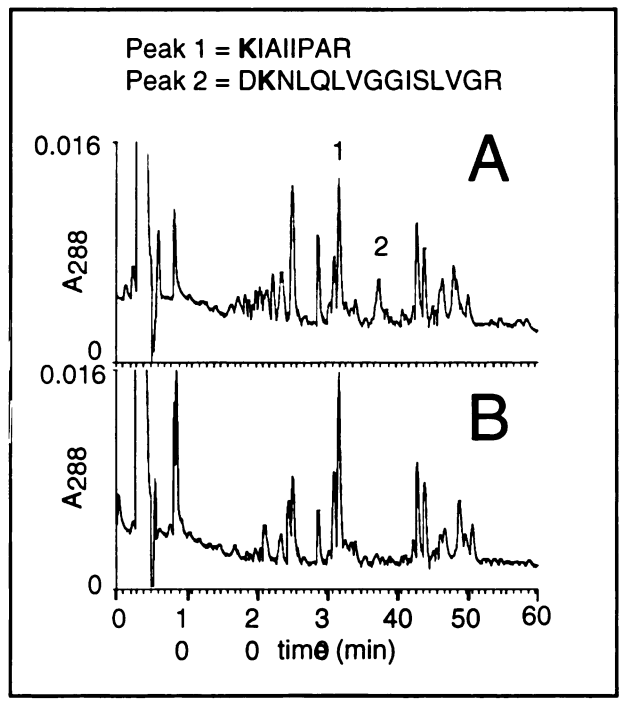


Fig. 4.8. **HPLC separation of the tryptic digest of CMP-NeuAc synthetase covalently modified by anhydro-ε-oC.** CMP-NeuAc synthetase was covalently modified by 0.1 mM anhydro-ε-oC in the absence (A) or presence (B) of 5 mM CTP for 100 min at 37°C in 0.1 M MOPS pH 7.1, 25 mM $MgCl_2$, 25 mM $NaBH_3CN$. The modified samples were treated as described in Fig. 4.7 to obtain tryptic peptides. Absorbance was monitored at 288 nm. Two peaks (labeled 1 and 2) were identified in the digest of enzyme modified by anhydro-ε-oC in the absence of CTP (A) that had a UV absorbance spectra characteristic of ε-cytidine. Peak 1 was also observed in the digest of enzyme modified in the presence of CTP (B), however, peak 2 was present only in A. The peptides identified in peaks 1 and 2 are listed above the chromatograms and covalently modified residues are shown in bold.

pmol glutamine in the last cycle. As for PLP modified lysine, no PTH derivative of anhydro-oC modified lysine was detected in cycle 2. The data for cycle three was lost due to a machine malfunction that did not affect the sequencing chemistry. The lack of a PTH-lysine in cycle 2, the molecular masses of the peptides labeled with anhydro-oC and anhydro-ε-oC, and the PSD fragmentation is strong evidence that K_{19} is covalently modified in these peptides.

4.4 DISCUSSION

In this study we have shown that a lysine conserved in CMP-NeuAc synthetases (K_{19} in the *H. ducreyi* enzyme) is covalently labeled by the aldehyde reagents anhydro-oC, anhydro-ε-oC, and PLP when the reaction is done in the absence of the substrate CTP, and is not labeled in the presence of CTP. There are two conserved lysine and four conserved arginine residues in the family of bacterial CMP-NeuAc synthetases (see Chapter 3). CMP-KDO synthetase catalyzes a very similar reaction to CMP-NeuAc synthetase, and these bacterial enzymes share some homology with CMP-NeuAc synthetases. When both CMP-NeuAc synthetases and CMP-KDO synthetases are compared, only one of the lysine and one of the arginine residues are conserved (K_{19} and R_{10} in *H. ducreyi* CMP-NeuAc synthetase). These two amino acids are found in a region of high sequence homology. In a recent report of a crystal structure of CMP-KDO synthetase, both of these residues were shown coordinating an $IrCl_6^{3-}$ anion in a large pocket, and were postulated to be involved in CTP binding (Jelakovic et al. 1996). A preliminary report has shown the importance of these two residues in CTP binding and enzyme activity of the *E. coli* CMP-NeuAc synthetase using site directed mutagenesis (Stoughton et al. 1997). There is a third conserved positively charged amino acid residue (lysine in CMP-NeuAc synthetases and arginine in CMP-KDO synthetases) found in the N-terminal region as well. This lysine (K_{14} in *H. ducreyi* CMP-NeuAc synthetase) was also modified by PLP. A peptide labeled

with PGO was not isolated, however the kinetics are consistent with as few as one arginine being involved in the loss of activity. Because CTP is capable of protecting the enzyme from PGO as well, it is highly likely that R_{10} is the residue involved. A positively charged residue at this position in the *E. coli* CMP-NeuAc synthetase was found to be necessary for enzyme activity and is likely involved in binding CTP (Stoughton et al. 1997).

Periodate oxidized nucleotides have been used, in this study and many others, as affinity labels to covalently modify the nucleotide binding sites of enzymes (see (Bazaes 1987; Colman 1990) for review). Although valued for the ease in which they are prepared and the relatively minor perturbation the modification has on the nucleotide structure, many have documented problems encountered with the use of these reagents (Lowe and Beechey 1982; Colman 1990; Lin and Chang 1993). In fact, there have been very few cases in which peptides modified by periodate oxidized nucleotides have been successfully isolated and characterized (Chan et al. 1985; Bezares et al. 1987; Low et al. 1993). We too had difficulties using oCTP to label CMP-NeuAc synthetase. Initial experiments demonstrated that β-elimination of triphosphate was occurring during the labeling reaction, as determined by ESI-MS (Fig. 4.3A). In solution, very little oCTP degraded to anhydro-oC under the standard labeling conditions (Table 4.2), indicating that the β-elimination reaction was occurring after modification of the enzyme. The maximum concentration of anhydro-oC in the 2.5 mM oCTP solution after 30 min would be 137.5 μM, with a calculated $t_{1/2}$ of 10.3 min. The calculated $t_{1/2}$ of 2.36 mM oCTP is 4.1 min. Clearly, oCTP and not contaminating anhydro-oC is responsible for most of the inactivation. Others have demonstrated this type of enzyme accelerated β-elimination reaction by measuring far greater incorporation of ^{14}C or ^{3}H compared to ^{32}P onto the enzyme using mixtures of radioactively labeled dialdehyde nucleotides (Lowe and Beechey 1982; King and Colman 1983).

Some studies using periodate oxidized nucleotides have provided evidence that the covalent modification proceeds by formation of a Schiff base, however, in a number of instances and in model reactions the data indicates that a morpholino structure is formed

(Hansske et al. 1974; Lowe and Beechey 1982; King and Colman 1983; Rayford et al. 1985; Colman 1990). Evidence favoring formation of a morpholino derivative instead of a Schiff base includes: 1) inability to reverse the inactivation or remove incorporated nucleotide by extensive desalting or dilution even without reduction (Gregory and Kaiser 1979; King and Carlson 1981), 2) no incorporation of ^3H when NaB^3H_4 is used to reduce the modified enzyme (King and Colman 1983; Mas and Colman 1983; White and Levy 1987), and 3) stability of the modification in dilute acid (Gregory and Kaiser 1979). In general, it seems that the morpholino derivatives are fairly stable under moderate conditions, but harsher treatment, such as SDS-PAGE or concentrated acid, can release or degrade the modification (Gregory and Kaiser 1979; King and Carlson 1981). This appears to be the case with the oCTP adduct formed with CMP-NeuAc synthetase. The modification survived extensive desalting, along with purification of the enzyme under the acidic (pH 2) and denaturing conditions of reverse phase HPLC. However, heating at 60°C in 6 M guanidinium chloride released most of the adduct. Furthermore, we were able to consistently recover a peptide from tryptic digests that appeared to be modified with a morpholino derivative of oCTP. Two ions were observed in MALDI-MS analysis (1775 Da and 1759 Da avg. mass) of the peptide fraction that varied in relative proportion between different digests of labeled enzyme. These masses can be explained by the covalent modification of tryptic peptide 18-32 with the non-reduced 2B (Fig. 4.4) and the partially reduced 3B that has lost H_2O, but has not been completely reduced. However, the yield of the labeled peptide was always unsatisfactorily low in comparison to the extent of inactivation and labeling of the enzyme, so further efforts were devoted to obtaining a more stable modification.

When $NaBH_3CN$ was included in the reaction of oCTP with CMP-NeuAc synthetase, the β-elimination reaction was largely avoided (Fig. 4.3B) and the mass for the covalent modification was consistent with the formation of a completely reduced morpholino adduct (4A in Fig. 4.4). This structure is analogous to the product of the model reaction of oAdo and glycine in the presence of $NaBH_3CN$ (Rayford et al. 1985).

Despite the formation of what should be a highly stable covalent modification, difficulties were encountered in purifying and detecting a labeled peptide(s). Because a product lacking the phosphate moiety was recovered from the initial oCTP labeling experiments, we hypothesized that anhydro-oC labeled peptides might be easier to purify and characterize.

Anhydro-oC was several times more reactive than oCTP at equivalent concentrations. Although the reaction of anhydro-oC with CMP-NeuAc synthetase appeared to follow saturation kinetics, it was difficult to measure the rate of inactivation past 8 mM to demonstrate this conclusively. Other 4',5'-anhydro dialdehyde nucleosides have also been shown to be more reactive than the corresponding dialdehyde nucleotides, but the mechanism was reported to be a simple irreversible reaction without a prior binding step (Lowe and Beechey 1982; Chang et al. 1990). However, in both cases the highest concentration of 4',5'-anhydro dialdehyde nucleoside tested was less than twice the K_I of the corresponding dialdehyde nucleotide, as was the case in this study. It is possible that a saturation effect would have been observed if higher concentrations of the anhydro compounds had been tested. In the presence of $NaBH_3CN$, the reaction of anhydro-oC with the enzyme produced a covalent modification with a mass consistent with the fully reduced morpholino derivative (4B in Fig. 4.4), analogous to the results obtained with oCTP.

The *H. ducreyi* CMP-NeuAc synthetase has a fairly strict requirement for CTP as its nucleotide substrate (Table 4.3). Nucleotides with variations in the base moiety were not used by the enzyme while those that varied at the 2' hydroxyl were only weak substrates. Ara-CTP and ε-CTP were both reported not to be substrates or inhibitors of the *E. coli* CMP-NeuAc synthetase (Vann et al. 1987). Furthermore, only CTP, and not 2'-deoxy-CTP or UTP, was capable of protecting the *H. ducreyi* CMP-NeuAc synthetase from inactivation by oCTP. In light of this high specificity for CTP, it was quite surprising to find that oUTP and even oATP inactivated the enzyme to the same extent as oCTP (Table 4.1). In contrast, Lowe and Beechey showed that oADP and oATP were much better inactivators of ATPase than either oCTP or oAdo (Lowe and Beechey 1982). However,

the amount of β-elimination product in the oUTP and oATP preparations was greater than for oCTP, particularly for the oUTP sample, complicating interpretation of the results (Table 4.2). Because anhydro-oC, and presumably other 4'5'-anhydro dialdehyde nucleosides, react much faster than the corresponding dialdehyde nucleotide, the β-elimination product in the oUTP and oATP samples may be responsible for most of the inactivation. Without purifying the oUTP and oATP samples and completing a more detailed kinetic analysis of the nucleotides and their β-elimination products, it is not possible to determine the extent of inactivation caused by oUTP and oATP. However, whether the inactivation is caused by the oxidized nucleotide or its β-elimination product or both, the reagent responsible is almost certainly not binding to the enzyme in the same manner as the substrate CTP.

This brings into question whether oCTP is really acting as a true affinity label. Although saturation kinetics were followed for the reaction of oCTP with CMP-NeuAc synthetase, and this is often taken as evidence of a preliminary non-covalent binding step occurring on the enzyme prior to reaction, another explanation may prove more likely. The protection from inactivation provided by CTP, and the lack of protection with 2'-deoxy-CTP and UTP, provides strong evidence that one or more residues in or near the CTP binding site is being modified by all the reagents tested. However, based on the following observations, it seems likely that oCTP and anhydro-oC are not binding to the enzyme in the same manner as CTP, but are acting as simple chemical inactivators of CMP-NeuAc synthetase that are modifying more reactive lysine residues: 1) The K_I for oCTP is nearly 6 mM while the apparent K_D for CTP is approximately 100 μM (N. Samuels, unpublished data). Similarly, much greater values have been obtained for the K_I of dialdehyde nucleotides compared to the K_D of the corresponding nucleotide in some other instances and the authors suggested that distortion of the nucleotide structure might cause the large decrease in binding affinity (Mas and Colman 1983; White and Levy 1987). 2) The K_I for anhydro-oC, a compound which would be expected to have much less affinity for the enzyme than oCTP, is only about twice as great as that for oCTP. 3) Anhydro-ε-oC is as

potent an inactivator as anhydro-oC despite the fact that ε-CTP is not a substrate. 4) Likewise, the β-elimination products of oUTP and oATP, and possibly oUTP and oATP themselves, rapidly inactivate the enzyme. 5) Lysine residues with increased reactivity, due to a lowered pK_a from their local chemical environment, can account for selective modification (Eyzaguirre 1987). Similar explanations have been put forward for the increased reactivity of arginine residues in active sites and their ability to be selectively modified (Borders and Riordan 1975; Patthy and Thesz 1980). 6) Finally, the saturation kinetics observed can be explained by the chemical reaction that occurs on the enzyme. With respect to the last point, the saturation kinetics sometimes observed for the reaction of α-dicarbonyl reagents, such as PGO, with enzymes could also be explained by a chemical equilibrium instead of a binding equilibrium (Baburaj and Durani 1991). For the oCTP reaction with CMP-NeuAc synthetase, instead of the first reversible step being non-covalent binding to the active site (reaction 2), it could correspond to the reversible formation of the dihydroxymorpholino structure initially formed:

$$E + oCTP \underset{k_{-1}}{\overset{k_1}{<\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!-\!\!>}} E\text{-}oCTP \text{ (dihydroxymorpholino adduct, 1A)} \qquad (5)$$

The k_{max} would correspond to the irreversible loss of H_2O followed by triphosphate (in the absence of $NaBH_3CN$) to form the morpholino adduct 2B, or the loss of H_2O followed by reduction (in the presence of $NaBH_3CN$) to form the reduced morpholino derivative 4A.

Arginine residues are commonly found in the recognition sites of enzymes that bind phosphorylated compounds, or other anionic compounds (Borders and Riordan 1975; Riordan et al. 1977), so it is not unexpected that PGO inactivates CMP-NeuAc synthetases (this chapter and (Zapata et al. 1995)). PGO is a highly selective reagent for arginine residues and has been used to probe for essential arginine residues in enzymes that bind phosphorylated substrates or cofactors (Takahashi 1968; Powers and Riordan 1975; Takahashi 1977). PGO can react with arginine residues in proteins to form 2:1 or 1:1 complexes, which can possibly undergo rearrangement reactions (Takahashi 1968; Borders

and Riordan 1975; Vandenbunder et al. 1981; Bjerrum 1989). In the model reaction of PGO and arginine, a 2:1 complex is formed that is stable under acidic conditions, but slowly decomposes under neutral or basic conditions (Takahashi 1968). However, evidence for 1:1 complexes of PGO with arginine residues in proteins has been reported in some cases (Borders and Riordan 1975; Vandenbunder et al. 1981). ESI-MS analysis of PGO modified CMP-NeuAc synthetase revealed a 1:1 complex (Fig. 4.3D).

In conclusion, inactivation of CMP-NeuAc synthetase by aldehyde reagents and PGO, the isolation of peptides modified on K_{19}, along with the presence of highly conserved lysine and arginine residues in the N-terminal region strongly support a role for these residues in binding CTP and possibly in catalysis. Mass spectrometric analysis of covalently modified intact protein and tryptic peptides allowed us to determine the nature of these covalent modifications. Finally, some problems in the use of periodate oxidized nucleotides were presented.

CHAPTER 5.

Characterization of the Lipooligosaccharides from CMP-NeuAc Synthetase and Sialyltransferase Deficient Mutants of *Haemophilus ducreyi*

5.1 INTRODUCTION

After cloning the CMP-NeuAc synthetase gene (*neuA*) (Chapter 3), our next goal was to construct an isogenic mutant of *H. ducreyi* with this gene inactivated. A mutation was constructed in *neuA* by insertion of an Ω-Km-2 cassette, by Professor Robert Munson's group at Children's Research Hospital Foundation in Columbus, Ohio. The construct was cloned into a suicide vector which was electroporated into *H. ducreyi* strain 35000-HP (human passaged strain 35000) to undergo allele exchange. A second open reading frame (orf) found 49 base pairs downstream of *neuA* had been tentatively identified as a sialyltransferase by our collaborators because of its weak similarity to the *E. coli* K92 polysialyltransferase and its proximity to *neuA*. This orf was also inactivated using the same approach as used for *neuA*.

Lipooligosaccharides from both of these isogenic mutants were isolated and characterized by SDS-PAGE, carbohydrate, and mass spectrometric analysis. The amount of NeuAc found as a component of the LOS was reduced to undetectable levels for the *neuA* mutant and to less than 4% of the level of the parent strain, 35000-HP, for the putative sialyltransferase mutant. Complementation of the defects was attempted by supplying *neuA* or the putative sialyltransferase on a pLS88 shuttle vector. LOS analysis revealed that the putative sialyltransferase mutant was complemented. Indeed, the levels of NeuAc containing LOS glycoforms were substantially greater than those of the parent strain. The *neuA* mutant was not complemented by this approach and it was hypothesized that the insertion in *neuA* was having a polar effect on the putative sialyltransferase gene

directly downstream. Therefore, a second mutation was constructed in *neuA* that was designed to prevent polar effects and an isogenic mutant constructed as for the other mutations. This mutant was successfully complemented by *neuA* on the pLS88 vector. We now have several *H. ducreyi* clones with varying levels of NeuAc in their LOS; two with undetectable levels, two with greatly reduced levels, and one with levels higher than the parent strain. These clones will now enable us to better determine the biological role of NeuAc in the pathogenesis of *H. ducreyi.*

5.2 MATERIALS AND METHODS

5.2.1 Materials

Glucosamine, NeuAc, and anhydrous hydrazine were from Sigma. Aqueous HF (48% w/v, ACS reagent) and 2,5-dihydroxybenzoic acid were from Aldrich. Acrylamide/Bis-acrylamide solution (40% w/v, 37.5:1 monomer to cross-linker), electrophoresis quality Tris, glycine, and SDS were from BioRad. Brain Heart Infusion broth and GCII agar base were from BBL. Dialysis tubing was from Spectra/Por. Constant boiling 6 N HCl was from Pierce.

5.2.2 Methods

5.2.2.1 *Growth of Bacteria*

H. ducreyi strains were grown on chocolate agar plates (made from GCII agar base, 1% (v/v) IsoVitaleX, 1% (w/v) hemoglobin) that were incubated at 35°C in 5% CO_2 for 48 hrs. Kanamycin (20 μg/ml) and/or 5-bromo-4-chloro-3-indolyl β-D-

galactopyranoside (X-gal) (40 μg/ml) were included as needed. For the isolation of LOS, bacteria were grown in 0.5 or 1 L volumes of Brain Heart Infusion (BHI) broth containing 25 μg/ml hemin, 1% (v/v) IsoVitaleX, and 5% (v/v) fetal bovine serum at 35°C with aeration. No antibiotics were used for broth grown organisms.

5.2.2.2 *Construction of CMP-NeuAc Synthetase and Putative Sialyltransferase Isogenic Mutants and Complementation of Defects*

Mutations were constructed in *neuA* and the putative sialyltransferase gene by insertion of an Ω-Km-2 cassette, by our collaborators at the Children's Research Hospital Foundation in Columbus, Ohio (Perez-Casal et al. 1991). The disrupted genes were cloned into a vector derived from pSE380 (Invitrogen; Carlsbad, CA) containing *trp'-'lacZ* (Bozue et al. 1998), and the construct transformed into the wild-type *H. ducreyi* strain 35000-HP by electroporation (Hansen et al. 1992). pSE380 is a ColE1-type vector and will not replicate in *H. ducreyi*. Strain 35000-HP was obtained from strain 35000 that was recovered after inoculation of a human volunteer during studies of the human model of chancroid (Spinola et al. 1996). Resistant clones were selected on chocolate agar containing kanamycin and isogenic mutants that had undergone allele replacement were distinguished from cointegrates by restreaking clones on chocolate agar containing X-gal and kanamycin as described in (Bozue et al. 1998). The *neuA* mutant was designated 1917 and the putative sialyltransferase mutant, 1934. *neuA* and the putative sialyltransferase gene were cloned into the shuttle vector pLS88 (Hansen et al. 1992) and transformed into the corresponding isogenic mutant to complement the defective gene. A second *neuA* mutation was generated to avoid problems with polar effects by inserting a nonpolar kanamycin cassette and an isogenic mutant (designated 2014) constructed as above. Mutants transformed with pLS88 containing a functional copy of *neuA* or the putative sialyltransferase are designated by a (+) after their numeric identifier (i.e. 1917(+), 1934(+), and 2014(+)).

5.2.2.3 *Isolation of Lipooligosaccharides (LOS)*

LOS was isolated using a modified version of the hot phenol-water extraction procedure (Westphal and Jann 1965; Johnson and Perry 1976; Apicella et al. 1994). Lyophilized bacterial cell pellets (140-320 mg) were ground to a course powder with a metal spatula in a 50 ml tube. The powder was suspended in 10 ml of 50 mM NaH_2PO_4, 5 mM EDTA, 1 mg/ml lysozyme, pH 7.0 and stirred for one hour at room temperature. The cells were sonicated (25 W) for 1-5 min and then 5 ml of 50 mM NaH_2PO_4, 45 mM $MgCl_2$, 4 mg/ml lysozyme, 30 µg/ml DNase I, 30 µg/ml RNase A, pH 7.0 was added. The suspension was incubated for 60 min at 37°C and for an additional 60 min at 60°C. The cells were sonicated again (25 W, 1 min), an equal volume (15 ml) of 90% (w/v) phenol preheated to 70°C was added, and the suspension was incubated at 70°C for 15 min with occasional vortexing to insure mixing. The tubes were chilled on ice for at least 15 min before centrifuging at 9000 x g for 90 min (0-4°C) to separate the phenol and water phases. The aqueous phase was removed and dialyzed against several changes of water over the course of one day using 1000 molecular weight cut-off dialysis tubing. The dialyzed LOS was lyophilized, suspended in a small volume of water (1-4 ml), and purified by two rounds of ultracentrifugation. The final LOS pellet was suspended in water, lyophilized, and stored in the freezer. Typical yields were 0.1-0.4% of the bacterial dry weight.

5.2.2.4 *Preparation of O-deacylated LOS*

To prepare a water soluble LOS species amenable to mass spectrometric analysis, O-acyl groups were removed by treatment with hydrazine (Helander et al. 1988). Anhydrous hydrazine (20-100 µl) was added to lyophilized LOS samples to give a concentration of 1-10 µg/µl of LOS. The reaction was heated at 37°C for 20 min with occasional vortexing. After chilling the reactions at -20°C for at least 10 min, chilled acetone (8-10 volumes) was slowly added to destroy the hydrazine and precipitate the O-

deacylated LOS. The precipitate was pelleted by centrifugation (14000 x g for 120 min, 0-4°C) and the supernatant carefully removed. The pellet was suspended in a second, equivalent amount of chilled acetone and the centrifugation repeated. After removing the supernatant the pellet was dissolved in a small volume of water and lyophilized.

5.2.2.5 *SDS-PAGE Analysis of LOS*

Aliquots of LOS suspended in H_2O were diluted to 7.5-30 ng/µl with BioRad Laemmli Sample Buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% bromophenol blue) with 2% β-mercaptoethanol. Typically, samples were diluted 7-700 fold with this buffer to obtain these concentrations. The samples were heated in a boiling water bath for 5 min and allowed to cool before loading 100-200 ng of LOS into the sample wells. A 15% or 16% acrylamide resolving gel with a 4% stacking gel (14 cm x 16 cm x 0.75 mm) was used to separate the LOS (Laemmli 1970). The gel did not contain SDS although the running buffer did as per the usual conditions. A current of 12 mA was applied while the samples migrated through the stacking gel and 15 mA for the resolving gel. The electrophoresis was stopped 20-30 min after the dye had migrated off the end of the gel.

LOS was visualized by silver-staining according to the method of Tsai and Frasch, with a few minor changes (Tsai and Frasch 1982). The overnight fixing step was shortened from overnight to 30 min and the first three 15 min washes were shortened to 10 min each.

5.2.2.6 *NeuAc and Glucosamine Analysis of O-deacylated LOS*

NeuAc was released from O-deacylated LOS by mild acid hydrolysis using standard conditions (Reuter and Schauer 1994). O-deacylated LOS (1-3 µl, 5-20 µg) was diluted to 30 µl with water, mixed with 10 µl of 0.4 M HCl, and heated at 80°C for 60

min. After cooling, 12 µl of 0.4 M NaOH was added and the samples were frozen until ready for analysis by high-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). The entire sample was injected into a 500 µl loop on a Dionex HPLC system with a CarboPac PA1 column (Dionex; 4 x 250 mm), guard column, and inline filter. The column was eluted with 0.4 M NaOH and the PAD waveform potentials were as follows: 0.05 V (0.4 sec), 0.75 V (0.2 sec), -0.15 V (0.4 sec). Because there is some degradation of NeuAc under the hydrolysis conditions, quantitation was performed by treating known quantities of NeuAc (100-6400 pmol) under the same hydrolysis conditions used for samples to prepare a calibration curve.

To determine the molar amount of O-deacylated LOS used for NeuAc quantitation, the glucosamine content of the O-deacylated LOS was determined. An identical aliquot of O-deacylated LOS as was used for NeuAc analysis (1-3 µl) was added to 100 µl of 6 N HCl and heated at 100°C for 4 hrs to liberate glucosamine. After cooling the solution was diluted to 1 ml and lyophilized. Before analysis, the samples were redissolved in 100 µl of H_2O and injected on the Dionex HPLC system as for NeuAc quantitation except that elution was performed isocratically with 16 mM NaOH. Quantitation of glucosamine was performed by preparing a calibration curve from injections of a glucosamine standard (500-8000 pmol) in 100 µl of H_2O. A gluosamine standard (5000 pmol) was treated in the same manner as the O-deacylated LOS samples to estimate the amount of losses due to degradation and sample handling.

5.2.2.7 *Matrix Assisted Laser Desorption Ionization-Mass Spectrometry (MALDI-MS) of O-deacylated LOS*

Prior to mass spectrometric analysis, O-deacylated LOS samples (5-50 µg) dissolved in H_2O were diluted 5-10 fold with 50% CH_3CN to give approximately 0.2-1 µg/µl of O-deacylated LOS. A small quantity (roughly 20-100) of Dowex 50X 100-200 mesh beads (NH_4^+ form) suspended in 50% CH_3CN was added to the O-deacylated LOS

solutions and the tubes were mildly agitated for several minutes to desalt the O-deacylated LOS. A 1 µl aliquot of this solution was mixed with 1 µl of 100 mM 2,5-dihydroxybenzoic acid (recrystallized from H_2O) in 50% CH_3CN, 1 µl of the mixture was spotted on the MALDI sample plate and allowed to air dry. For analysis of the samples, a Voyager-DE time-of-flight (TOF) mass spectrometer with a nitrogen laser (337 nm) was operated in the negative-ion mode using an accelerating voltage of 20 kV, a grid voltage of 93%, a guide wire voltage of 0.05%, and a delay time of 200 nsec. The instrument was calibrated externally using bovine insulin B-chain (oxidized) (average $[M-H]^-$ = 3494.9 Da) and ACTH 1-24 (average $[M-H]^-$ = 2932.5 Da).

5.3 RESULTS AND DISCUSSION

Construction of H. ducreyi Mutants. After identifying and characterizing the CMP-NeuAc synthetase gene (Chapter 3), our collaborator Professor Robert Munson attempted to construct an isogenic mutant with *neuA* insertionally inactivated employing a protocol that had been used with success in his laboratory to create an isogenic hemolysin deficient mutant (Palmer et al. 1996). The method involves inserting an Ω-Km-2 cassette into the cloned gene of interest, cloning the construct into a suicide vector, and electroporating this vector into *H. ducreyi.* Isogenic mutants which have undergone allele exchange are selected on chocolate agar containing kanamycin. Unfortunately, Dr. Munson has found that this technique does not work well for some genes as *H. ducreyi* readily forms cointegrates which will also grow on the kanamycin containing media (Bozue et al. 1998). Several observations by his laboratory allowed them to develop a technique for resolving cointegrates from isogenic mutants using *lacZ* as a counter-selectable marker (Bozue et al. 1998). This method was used to construct mutants of both *neuA* and a second gene found 49 base pairs downstream of *neuA*. This gene was thought to possibly encode a sialyltransferase because of weak similarity to the *E. coli* K92

polysialyltransferase (Vimr et al. 1992) and its proximity to *neuA*. As mentioned in Chapter 1 (section 1.9), LPS biosynthetic genes are often arranged in clusters. Furthermore, genes for synthesis of the polysialic acid capsule of *E. coli* are arranged in a cluster and *neuS*, the polysialyltransferase, is the third orf downstream from *neuA* (Vann et al. 1993). Recently, enzyme activity data obtained in Dr. Munson's laboratory strongly suggests that this gene encodes the *H. ducreyi* LOS sialyltransferase (data not shown). Complementation of the mutants was attempted by transforming with *neuA* or the sialyltransferase gene cloned into the shuttle vector pLS88 as described previously for the hemolysin mutant (Hansen et al. 1992; Palmer and Munson 1995). SDS-PAGE analysis (see below) of the LOS from the *neuA* mutant indicated that the mutation was not complemented by this approach which suggested that the mutation was having a polar effect on the sialyltransferase downstream. A second mutation in *neuA* was constructed that was designed to prevent this polar effect.

Analysis of LOS by SDS-PAGE. The first method used to analyze the LOS from these mutants was SDS-PAGE (Fig. 5.1). The profiles of LOS bands from wild-type strains and mutants are very similar except for the band labeled Aa which is missing from all three mutants (2014, 1934, and 1917). Furthermore, the band reappears in 2014(+) and 1934(+), but not in 1917(+). In fact, the band is more intense in 1934(+) (relative to band A) than in the parent strain, 35000-HP. The LOS from *H. ducreyi* strain 35000, along with other strains, has previously been characterized by SDS-PAGE and the relative mobility of the NeuAc containing LOS band is known (Melaugh et al. 1994; Melaugh et al. 1996). The major LOS species, band A, is the LOS glycoform recognized by MAb 3F11 and contains the oligosaccharide whose structure was previously identified in our laboratory as the major oligosaccharide from strain 35000 LOS (see Chapter 1, Fig. 1.3 and Fig. 5.2) (Melaugh et al. 1994). Neuraminidase treatment of *H. ducreyi* LOS strongly supports that band Aa is the NeuAc containing LOS glycoform (Melaugh et al. 1996). The O-deacylated LOS of a *H. ducreyi* wild-type strain and an isogenic mutant whose major LOS glycoforms migrate at the same mobility as band B and band D, respectively, have

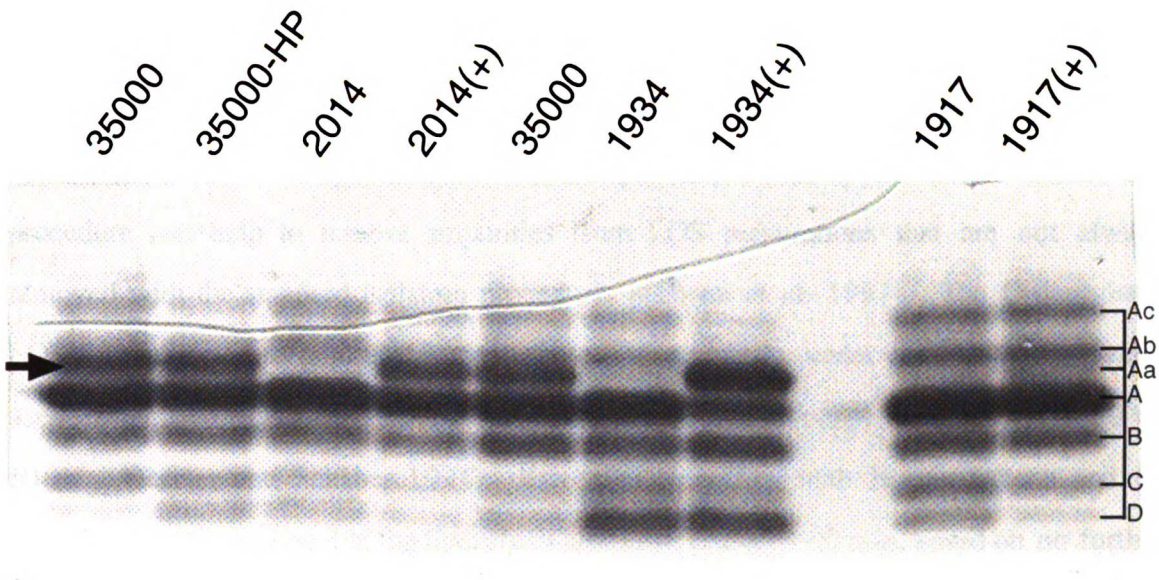


Fig. 5.1. **SDS-PAGE analysis of LOS from *H. ducreyi* wild-type strains, isogenic mutants, and complemented clones.** LOS (100-200 ng) was separated on a 16% acrylamide gel and silver-stained. Only the bottom portion of the gel where LOS bands were observed is shown. The arrow indicates the NeuAc containing LOS glycoform that is absent in the three mutants and restored in 1934(+) and 2014(+). The letters to the right refer to the proposed glycoforms to which the bands are believed to correspond to (see Fig. 5.2). The separation essentially results in a "ladder" of LOS glycoforms that differ by one monosaccharide. Although the separation is largely based on molecular weight, it is likely that the NeuAc containing species Aa has a somewhat faster mobility than expected because of the additional negative charge NeuAc adds to the LOS. The scanned image of the gel has been enlarged to 150%.

been characterized in our laboratory (data not shown) and supports the assignment of these bands to the structures shown in Fig. 5.1 as well.

NeuAc Analysis of O-deacylated LOS. Prior to carbohydrate and mass spectrometric analysis, the LOS was O-deacylated with anhydrous hydrazine. This procedure results in an LOS species that contains only two, N-linked fatty acid chains on the lipid A moiety (see Chapter 1, Fig. 1.2 for lipid A structure), which makes it far more water soluble and directly amenable to mass spectrometric analysis. Furthermore, this procedure can help to remove impurities from LOS preparations that are not always removed with the standard isolation procedures (Gibson et al. 1997). The O-deacylated LOS was subjected to mild acid hydrolysis to liberate NeuAc under conditions commonly used for the hydrolysis of NeuAc from glycoproteins and glycolipids (0.1 M HCl, 80°C, 60 min) (Reuter and Schauer 1994). Time course studies with NeuAc-lactose and O-deacylated LOS showed that the hydrolysis was complete after 60 min, based on no further increase in NeuAc observed beyond this time (data not shown). The results of NeuAc analysis of the O-deacylated LOS from wild-type strains (35000 and 35000-HP), isogenic mutants, and their complemented clones are given in Table 5.1. The mole % of NeuAc was determined by quantitation of the amount of glucosamine in an identical aliquot of O-deacylated LOS and assumes 3 moles of glucosamine per mole of O-deacylated LOS (one glucosamine from the oligosaccharide and two from the conserved lipid A core). These results confirm the qualitative SDS-PAGE analysis. Both of the *neuA* mutants, 1917 (polar) and 2014 (nonpolar), had undetectable levels of NeuAc in their O-deacylated LOS. As mentioned above, the first *neuA* mutant (1917) did not appear to be complemented by *neuA* on the shuttle vector pLS88 when its LOS was analyzed by SDS-PAGE. Indeed, 1917(+) had less than 10% of the NeuAc of the parent strain, 35000-HP. The sialyltransferase mutant (1934) had less than 4% of the NeuAc of the parent strain. In good agreement with the SDS-PAGE results, 1934(+) had the greatest amount of NeuAc of all the clones studied. The level of NeuAc in strain 35000 agreed well with results

Table 5.1. NeuAc and glucosamine content of O-deacylated LOS

Strain	NeuAc	$GlcNH_2$ [a]	LOS [b]	NeuAc/LOS [c]
	(pmol)	*(pmol)*	*(pmol)*	*(mole %)*
35000-HP	860	7230	2410	36
35000	670	8940	2980	22
1917	0	5750	1920	0
1917(+)	90	7780	2590	3
2014	0	2360	790	0
2014(+)	190	2790	930	20
1934	30	6620	2210	1
1934(+)	1430	7400	2470	58

[a] Glucosamine content was corrected by a factor of 1.14 to account for losses due to hydrolysis and sample handling. Glucosamine (5 nmol) treated under the same conditions as O-deacylated LOS gave 4480 ± 80 pmol and glucosamine (5 nmol) in H_2O gave 5090 ± 50 pmol (given as the mean of three measurements ± the standard deviation).

[b] O-deacylated LOS was calculated based on 3 moles of glucosamine per mole of O-deacylated LOS.

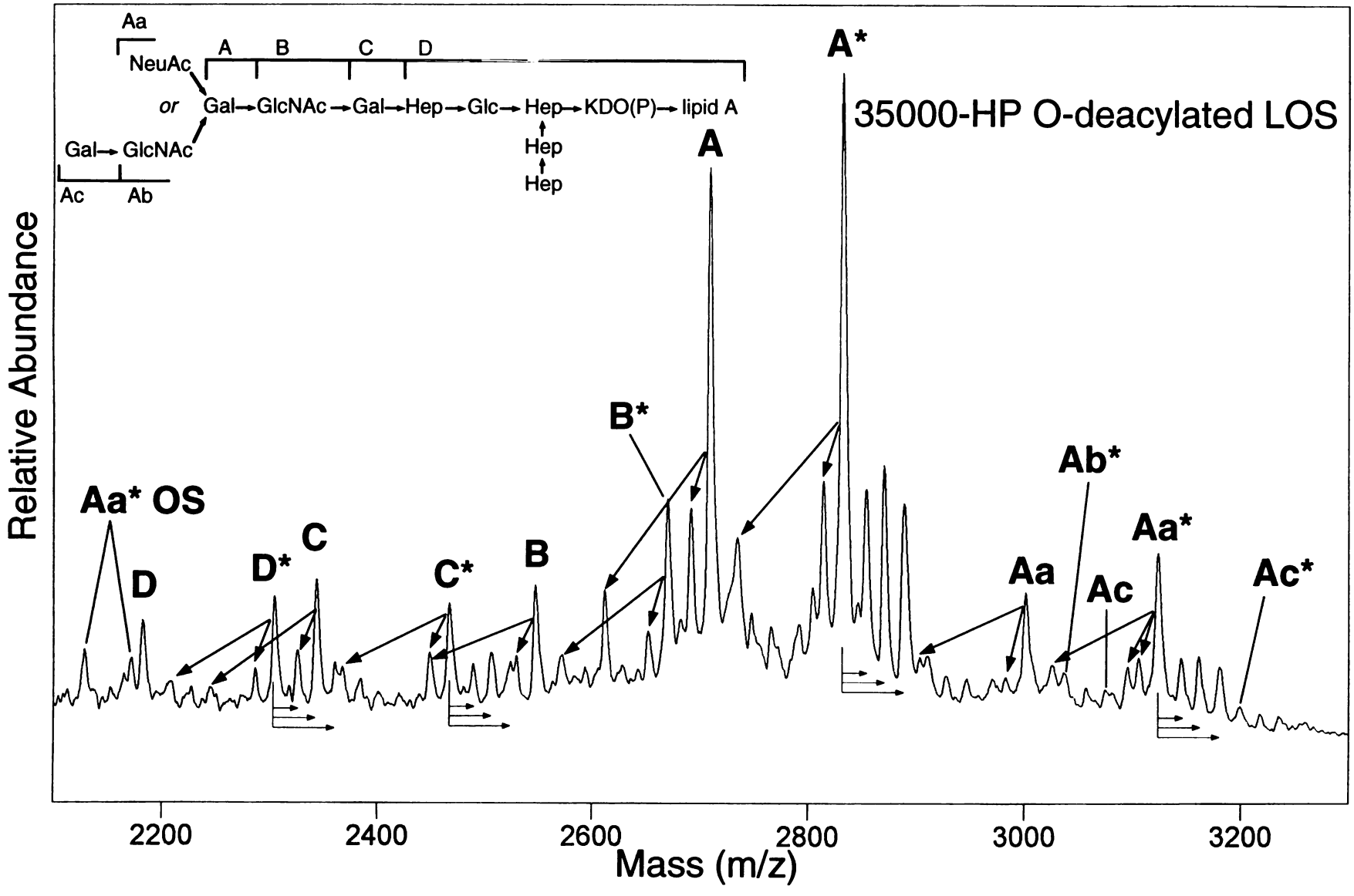
[c] Moles of NeuAc per mole of O-deacylated LOS.

previously obtained for this strain (Melaugh et al. 1996). Interestingly, the level of NeuAc in the LOS of 35000-HP was somewhat higher than for strain 35000.

MALDI-MS of O-deacylated LOS. The MALDI-TOF spectra of the O-deacylated LOS from *H. ducreyi* strain 35000-HP, the parent strain in this study, is shown in Fig. 5.2. As was also observed by SDS-PAGE, the LOS preparation is clearly heterogeneous. Mass spectrometry reveals a further level of heterogeneity; in addition to differences in the number of sugar residues in the oligosaccharide (Aa, Ab, A, B, C, and D in Fig. 5.2) each of these species may also be present with a phosphoethanolamine (PEA) moiety. The largest peaks, A* and A, correspond to the O-deacylated LOS species (with and without PEA) containing the major oligosaccharide structure from strain 35000 (Melaugh et al. 1994). Likewise, peaks Aa* and Aa correspond to the addition of NeuAc to A* and A, respectively (Melaugh et al. 1994; Melaugh et al. 1996). The relative intensities of the different LOS glycoforms agrees well with the qualitative analysis by SDS-PAGE. As has been observed previously by our laboratory in MALDI-MS analysis of O-deacylated LOS, MALDI generated prompt fragments corresponding to the loss of H_2O and H_3PO_4 are readily apparent in the spectrum (Gibson et al. 1997). Fragmentation between the O-deacylated lipid A moiety and the oligosaccharide are also readily observed with this technique and can be quite useful for identification of components. Adducts, particularly of the PEA containing species, are also commonly observed and can be seen in Fig. 5.2 as well (Gibson et al. 1997).

The MALDI-TOF spectra of the O-deacylated LOS from the three isogenic mutants (2014, 1934, and 1917) compared to the parent strain are shown in Fig. 5.3. Clearly, there is no evidence of peaks corresponding to a NeuAc containing glycoform (Aa* or Aa). Otherwise, all four spectra appear very similar. Interestingly, the 1934 mutant has a greater proportion of the D* and D components than the other mutants and the parent strain which was also evident by SDS-PAGE. The MALDI-TOF spectra of the O-deacylated LOS from the complemented clones compared to the parent strain are shown in Fig. 5.4. Peaks for the NeuAc containing LOS glycoforms, Aa* and Aa, are present at levels comparable to the

Fig. 5.2. **MALDI-TOF spectrum of O-deacylated LOS from *H. ducreyi* 35000-HP.** The spectrum is quite similar to that obtained for strain 35000 previously by our laboratory, although there are more prompt fragments and adducts in this spectrum (Gibson et al. 1997). All of the major peaks, and most of the minor peaks, have been assigned and are based on previous characterization of the LOS from strain 35000 and other wild-type strains by our laboratory (Campagnari et al. 1994; Melaugh et al. 1994; Melaugh et al. 1996; Gibson et al. 1997). The letters refer to the structure in the top left corner, and an asterisk indicates the addition of a PEA moiety. Lipid A refers to O-deacylated lipid A in this figure. Prompt fragments which correspond to the loss of H_2O and H_3PO_4 are shown by the large, downward pointing arrows. A loss of 28 Da is also observed from Aa* which is due to loss of CO from the NeuAc containing glycoform. A number of adducts were observed at approximately +22 ($[M-2H+Na]^-$), +38 ($[M-2H+K]^-$), and +57 Da, primarily from PEA containing species and are indicated by the small, horizontal arrows. It is not known what this adduct is, but it could be Co^{2+} or Ni^{2+}, which would give adducts of 56.9 and 56.7 Da, respectively. The average mass shift is 56.7 Da with a standard deviation of 0.5 Da measured from the A* and Aa* peaks in the eight separate O-deacylated LOS samples analyzed in this study. The +57 Da adducts are in all the samples prepared for this study. Although we have not observed this adduct before, we have observed a number of different adducts from glycoforms containing PEA. It is thought that these PEA-containing glycoforms bind metal ions very tightly because the PEA is bound to phosphate, forming a pyrophosphate linkage (Gibson et al. 1997). Two fragments corresponding to cleavage of the Aa* O-deacylated species into oligosaccharide (Aa* OS) and lipid A moieties are observed at the lower end of the mass scale. The species lower in mass is due to the loss of CO_2 (44 Da) from the KDO of the oligosaccharide. Other oligosaccharide and lipid A fragments are not shown here because they are off scale at lower mass, but some are shown in Figs. 5.3 and 5.4.


Aa
NeuAc
A
B
C
D
or
Gal→GlcNAc→Gal→Hep→Glc→Hep→KDO(P)→ lipid A
Hep
Hep
Gal→ GlcNAc
Ac
Ab
35000-HP O-deacylated LOS
Relative Abundance
A*
A
B*
Ab*
Aa* OS
D
D*
C
C*
B
Aa
Ac
Aa*
Ac*
2200
2400
2600
2800
3000
3200
Mass (m/z)

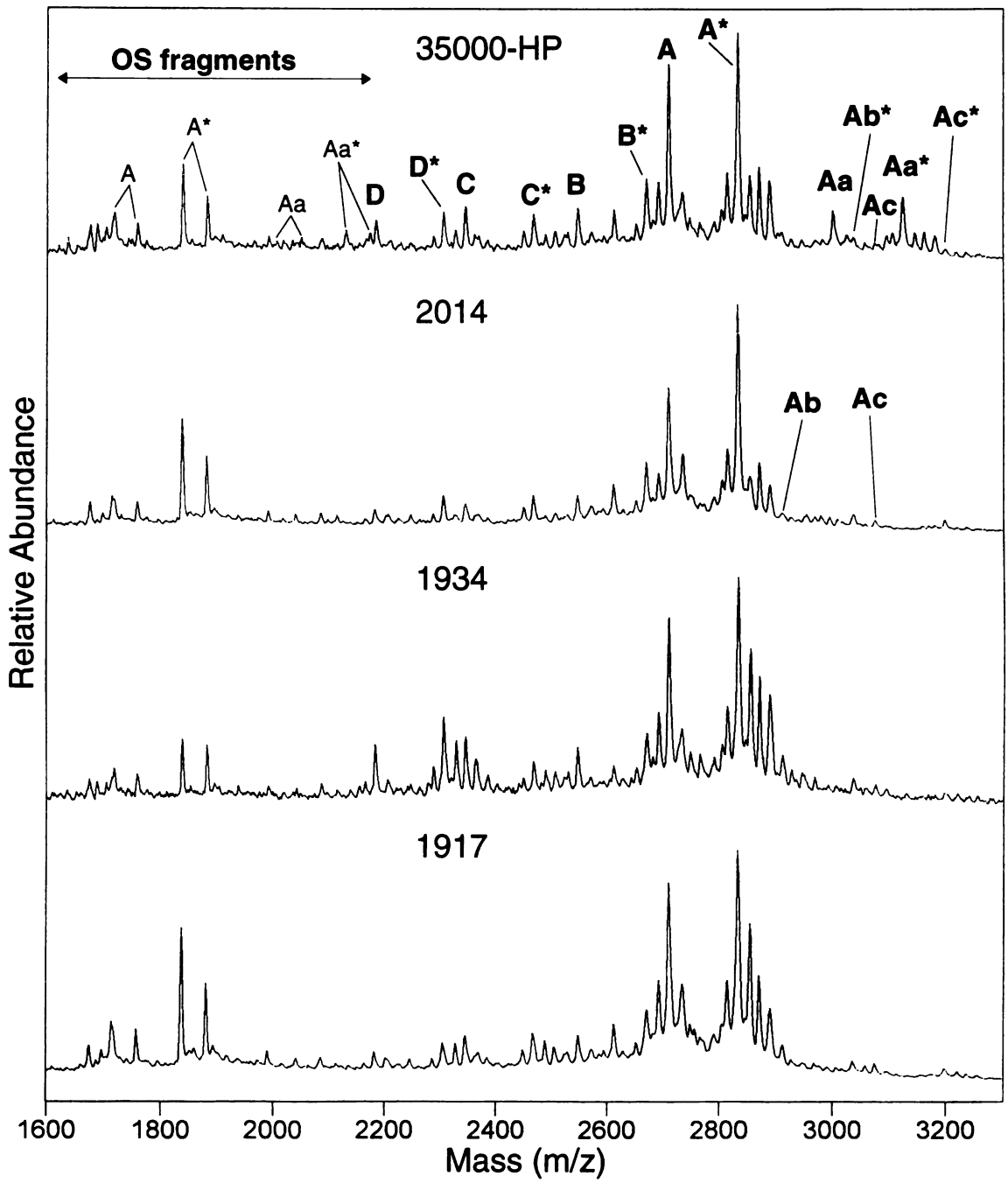


Fig. 5.3. **MALDI-TOF spectra of O-deacylated LOS from *H. ducreyi* isogenic mutant strains compared to 35000-HP.** The spectrum of 35000-HP is the same as in Fig. 5.2 and is shown here for comparison. All four spectra are quite similar, except for the absence of NeuAc containing glycoforms (Aa and Aa*) from all three of the mutants. Glycoform Ab was not clearly observed in 35000-HP and so is labeled in 2014. Oligosaccharide is abbreviated as OS.

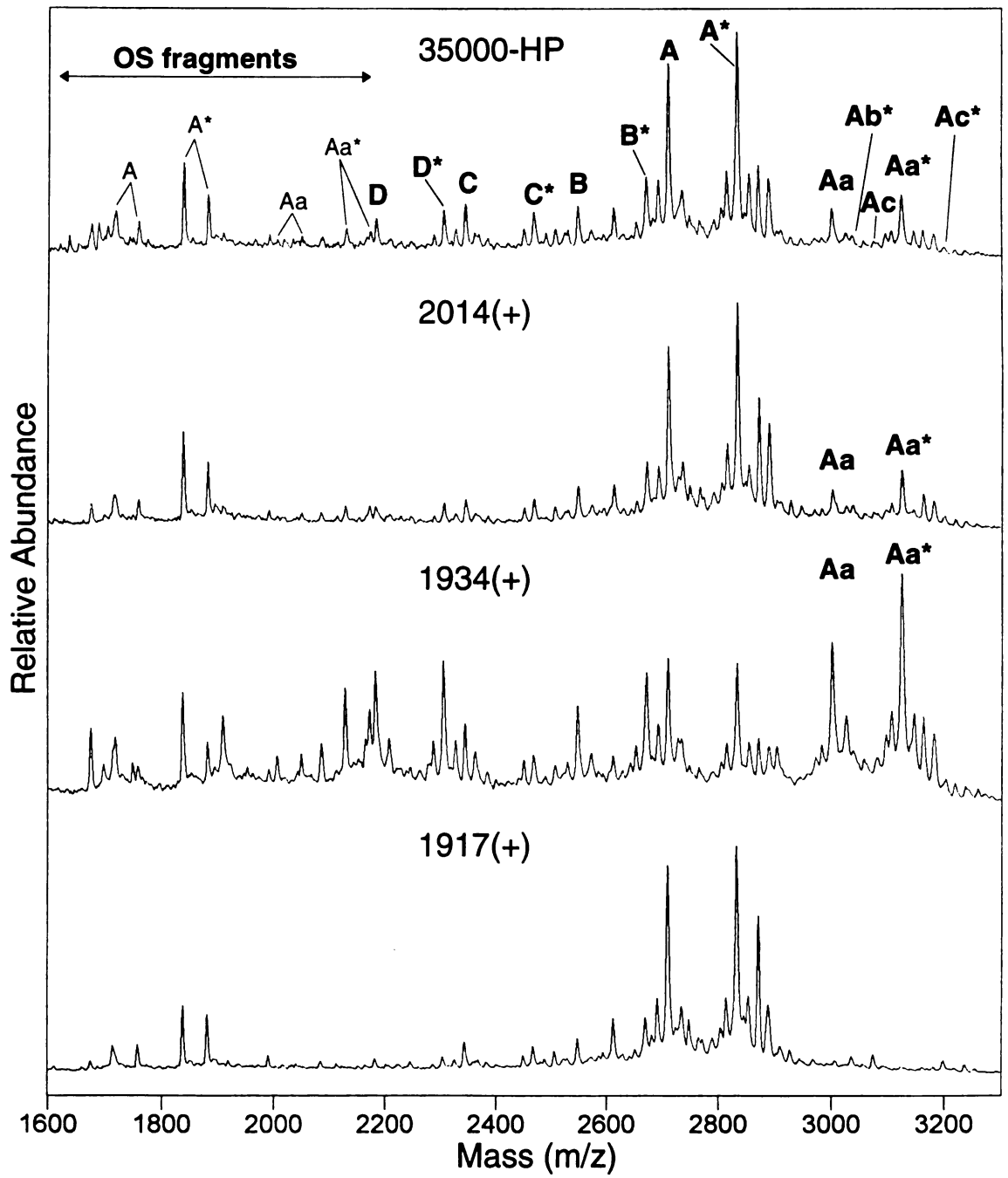


Fig. 5.4. **MALDI-TOF spectra of O-deacylated LOS from *H. ducreyi* complemented mutant strains compared to 35000-HP.** The spectrum of 35000-HP is the same as in Fig. 5.2 and is shown here for comparison. The NeuAc containing glycoforms (Aa and Aa*) are seen in both 2014(+) and 1934(+), but not 1917(+). The increase in the amount of Aa and Aa* glycoforms in 1934(+) compared to 35000-HP is quite dramatic.

Table 5.2. MALDI-MS analysis of O-deacylated LOS from *H. ducreyi* wild-type strains, isogenic mutants, and complemented clones

calc. [M-H]⁻ [a]	Observed [M-H]⁻ [a]								LOS glycoform [b]
	35000-HP		35000		2014(+)		1934(+)		
		(%) [c]		(%)		(%)		(%)	
2183.0	2182.0	18	2182.8	12	2183.3	11	2182.4	59	D
2306.0	2304.9	21	2306.1	11	2305.9	12	2305.4	63	D*
2345.1	2344.0	24	2344.8	24	2344.8	14	2344.0	37	C
2468.2	2467.2	20	2468.5	22	2468.2	14	2468.0	23	C*
2548.3	2547.0	23	2547.8	23	2548.2	20	2547.6	44	B
2671.4	2670.1	36	2670.9	30	2671.2	30	2670.8	58	B*
2710.5	2709.3	86	**2710.1**	**100**	2710.4	81	2709.6	65	A
2833.5	**2832.5**	**100**	2833.5	85	**2833.6**	**100**	2833.1	62	A*
3001.7	3001.0	22	3002.0	18	3002.1	18	3001.2	71	Aa
3124.7	3123.9	28	3125.4	18	3125.0	27	**3124.3**	**100**	Aa*
2913.7	ND [d]	--	2912.5	12	ND		ND	--	Ab
3036.7	3036.8	10	3038.3	9	3037.8	11	ND	--	Ab*
3075.8	3074.5	7	3075.9	8	3075.2	8	ND	--	Ac
3198.9	3199.2	5	3201.3	4	3199.5	7	ND	--	Ac*
	1917		1917(+)		2014		1934		
		(%)		(%)		(%)		(%)	
2183.0	2181.9	12	2182.0	6	2182.1	10	2182.3	26	D
2306.0	2305.1	16	2305.0	7	2305.1	16	2305.2	39	D*
2345.1	2344.1	19	2344.0	14	2344.7	12	2343.8	30	C
2468.2	2467.7	20	2467.1	12	2466.9	16	2467.9	19	C*
2548.3	2547.4	19	2547.2	16	2547.3	16	2547.6	25	B
2671.4	2670.3	30	2670.3	25	2670.3	31	2671.2	31	B*
2710.5	2709.5	85	2709.4	92	2709.4	64	2709.9	82	A
2833.5	**2832.7**	**100**	**2832.6**	**100**	**2832.6**	**100**	**2833.1**	**100**	A*
3001.7	ND	--	ND	--	ND	--	ND	--	Aa
3124.7	ND	--	ND	--	ND	--	ND	--	Aa*
2913.7	2911.8	15	ND	--	2911.7	8	2911.9	22	Ab
3036.7	3036.2	8	3036.2	8	3036.2	8	3036.9	12	Ab*
3075.8	3074.7	7	3074.5	8	3074.5	5	3075.5	9	Ac
3198.9	3198.2	3	3198.1	6	3198.3	5	3199.0	6	Ac*

[a] The calculated and observed [M-H]⁻ are reported as the average mass. The ion of greatest abundance is listed in bold. Spectra are shown in Figs. 5.2-4 (except for 35000).

[b] See Fig. 5.1 for the possible LOS glycoforms. An asterisk indicates the addition of a PEA moiety. Aa and Aa* are the glycoforms that contain NeuAc.

[c] Relative % abundance as determined by peak heights.

[d] ND, not detected.

parent strain in 2014(+) and are strikingly greater in 1934(+). As expected from the SDS-PAGE and NeuAc analysis of 1917(+), no NeuAc containing glycoforms were detected. A comparison of the glycoforms observed in the MALDI experiments and their relative abundance are shown in Table 5.2.

In conclusion, SDS-PAGE, carbohydrate composition, and mass spectrometric analysis of the LOS isolated from CMP-NeuAc synthetase and sialyltransferase deficient isogenic mutants showed complete, or near complete, lack of NeuAc incorporation. These results are consistent with these genes being essential for sialylation of the LOS as would be expected based on the proposed biosynthetic pathway (Chapter 1, Fig. 1.4). Successful complementation of these mutations has demonstrated that only the insertionally inactivated gene in question (*neuA* or sialyltransferase) is responsible for the abolition of sialylation. Studies to assess the role of NeuAc in the pathogenesis of *H. ducreyi* using these mutants is currently underway. These mutants will be tested in both the swine (Hobbs et al. 1995) and human (Spinola et al. 1996) disease models for any change in their ability to cause ulceration (swine) and/or pustules (human and swine) compared to the parent strain. Also, the effect that the absence of NeuAc has on the organism's ability to resist serum and phagocytic killing will be investigated. As NeuAc and galactose (now completely exposed as the terminal sugar in the LOS of these mutants) have well known roles in adhesion (see Chapter 1, sections 1.8 and 1.10), the ability of these mutants to adhere to and invade human keratinocyte and other cell lines will also be studied. As a result of our studies on the CMP-NeuAc synthetase of *H. ducreyi*, we hope to have a better molecular and biological understanding regarding the role of sialylation in the pathogenesis of this organism.

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