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

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An N-acetylneuraminic acid cytidylyltransferase (EC 2.7.7.43) (CMP-NeuAc synthetase) was isolated from a Haemophilus ducreyi strain 35000 cell lysate and partially characterized. The enzyme catalyzes the reaction

\[
\text{CMP-NeuAc} + \text{pyruvate} \rightarrow \text{NeuAc} + \text{P, (NeuAc aldonase)}
\]

REACTION Ib

The following reactions (I–III) are involved in the biosynthesis of sialic acid containing molecules and are catalyzed by the enzymes shown in parenthesis to the right. In the final step, the hydroxyl group of a carbohydrate serves as the acceptor (HO acceptor) for the nucleotide-sialic acid donor, CMP-NeuAc:

\[
\text{ManNAc} + \text{PEP} \rightarrow \text{NeuAc} + \text{P, (NeuAc synthetase)}
\]

REACTION Ia

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Sialic acid can be synthesized in bacteria by condensation of N-acetylmannosamine with either phosphoenolpyruvate (Ia) or pyruvate (Ib). The first reaction is catalyzed by NeuAc synthetase, which has been partially purified from N. meningitidis (27). This enzyme differs from the mammalian enzyme which synthesizes NeuAc-9-P (from N-acetylmannosamine-6-P and phosphoenolpyruvate), which is then dephosphorylated by a phosphophorylase. Recently, it was demonstrated that NeuAc synthesis in E. coli K1 occurs only through NeuAc aldolase (Ib) (28). CMP-NeuAc synthetases catalyze the second step (II) in the pathway, forming the nucleotide-sugar donor used by sialyltransferases. These enzymes have been purified and characterized from several bacterial and animal sources (29–33). A LOS-specific sialyltransferase has been detected in extracts of Neisseria sp. and partially characterized (34). To date, no bacterial LOS-specific sialyltransferase has been purified to homogeneity or cloned, although a recent report characterizing a bacterial LOS-specific sialyltransferase has been published (35). In contrast, bacterial polysialyltransferases have been purified and characterized from a number of bacterial species (36). As a group, these enzymes may be attractive targets for therapeutic intervention considering the role of sialic acid containing molecules as virulence and/or adhesion factors. In this report we detail the isolation of the CMP-NeuAc synthetase from H. ducreyi, the cloning of its gene, and expression of the enzyme in E. coli using a T7lac promoter system. Moreover, sequence analysis of CMP-NeuAc and CMP-KDO synthetases reveal a number of residues and domains conserved throughout both enzyme classes.

EXPERIMENTAL PROCEDURES

Materials

Reactive Green 19-Agarose, Cibacon Blue 3GA-Agarose, CTP, N-acetylneuraminic acid, N-glycolyneuraminic acid, 2-keto-3-deoxy-D-manno-octulosonic acid, ampicillin, kanamycin, and SDS-PAGE molecular weight standards were from Sigma. Size exclusion chromatography standards were from Sigma and Bio-Rad. Isopropyl β-D-thiogalactoside was from Life Technologies, Inc. H. ducreyi strain 35000 is a typed CDC strain and was a generous gift of Dr. A. Campagnari (SUNY at Buffalo).

Methods

Growth of Bacteria—H. ducreyi

The bacterial suspension (3 g of wet weight cells in 20 ml of 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 × g for 10 min. The supernatant was applied to two 5-ml EconoPac Q cartridges (Bio-Rad) 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 Cibacon 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 (Bio-Rad; 7.5 × 600 mm each) size exclusion columns connected in series and equilibrated with 20 mM KH2PO4, 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 column (Pharmacia Biotech Inc., 5 × 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 (NH4)2SO4. The sample was applied to a Hydrosphere-5-HIC column (Rainin; 46 × 100 mm) and eluted with 0.1 M KCl. The enzyme was purified further with a gradient of 0.5 M KH2PO4, 0.5 mM EDTA, pH 7.0 (buffer B), and eluted with a gradient of 0.1 M KH2PO4, 0.5 mM EDTA, pH 7.0 (buffer C) (0.5 ml/min, 1.2–0 M (NH4)2SO4 in 40 min). The active fractions (3 ml) were readjusted to 1.2 M (NH4)2SO4 and rechromatographed on the HIC column using a shallower gradient (0.4 ml/min, 1.125–0.6 M (NH4)2SO4 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 × 150 mm). The C4 column was equilibrated with 0.1% trifluoroacetic acid, and the proteins were eluted with a gradient of 0.08% trifluoroacetic acid, 70% CH3CN over 70 min at a flow rate of 0.2 ml/min.

Sialic 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 phenylthiohydantoin analyzer. For the fraction identified as containing the CMP-NeuAc synthetase, 43 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%), approximately 10–13 pmol (0.25–0.33 μg) of CMP-NeuAc synthetase was loaded on the sequencer.

Screening of the H. ducreyi Genomic DNA Library—The NH2-terminal amino acid sequence was reverse-translated employing the CompGene program (38). Two degenerate oligonucleotides were constructed (sequences 1 and 2). Oligonucleotide 1, a 23-mer containing a mismatch 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 1×DASHII library of H. ducreyi 35000 DNA (39) was plated, plaques were transferred to Hybond-N, and DNA was immobilized by UV cross-linking (Stratalinker) employing standard methodologies. Oligonucleotide 1 was end-labeled with γ-32P using T4 polynucleotide kinase, and hybridization was performed overnight in 5 × SSPE (1 × SSPE = 0.18 M NaCl, 0.01 M sodium phosphate, and 1 mM EDTA, pH 7.0), 5 × Denhardt’s solution, 0.5% SDS, and 20 μg/ml Salmon testes DNA (10 μg/ml). Following hybridization, the membranes were hybridized three times at 35°C in 2 × SSPE-0.1% SDS. The membrane was then washed in Saran Wrap and autoradiographed. Phage plaques, which gave a positive hybridization signal, were picked and rescreened. Phage DNA was isolated from 10 ml of liquid lysates and characterized by restriction analysis.

Cloning and DNA Manipulations—The low copy number vector pWSK30 was employed for the initial subcloning (40). For subsequent studies, the DNA fragment containing CMP-NeuAc synthetase was amplified by 20 rounds of PCR using primers 3 and 4 (see Table I). The PCR product was cloned into the TA donor cloning kit according to the manufacturer’s instructions (Invitrogen). Plasmid vectors pBT7-7, pBT4 (41) and pET24a (Novagen) were employed for the amplification of the OmpC expression constructs. Plasmid DNA was purified by using the Qiawell-8 plasmid kit (Qiagen, Chatsworth, CA).

DNA sequence was determined by the dideoxy chain termination method employing Sequencing and the manufacturer’s directions (U. S. Biochemicals Corp.). Lasergene software (DNASTAR, Madison, WI) was employed for data assembly and sequencing.

Protein Sequence Alignment—Protein sequence data bases (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 (42), 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 Ref. 43. In pairwise comparisons, the smaller of the two sequences was taken as the denominator.

Protein Expression—E. coli BL21(DE3) harboring the pET24 expres-
sion construct containing the CMP-NeuAc synthetase (designated pMV1) was grown at 30°C in LB broth containing 50 μg/ml kanamycin. After reaching an A600 between 0.3 and 0.8, the culture was induced with 0.1 mM isopropyl β-D-thiogalactopyranoside for 10 h at 30°C. The cells were harvested and frozen at −80°C until needed.

Purification of Recombinant CMP-NeuAc Synthetase—The cell paste (11 g) from a 3-liter culture was suspended in 40 ml of 50 mM Tris-HCl, 0.1 mM NaCl, pH 7.5 (buffer D), and the cells were lysed by two passages through a French press (approximately 20,000 psi). The lysate was sonicated (four 30-s pulses at 25 watts with 2-min pauses) to shear nucleic acids and reduce its viscosity. The lysate was then centrifuged at 15,000 × g for 90 min to remove cellular debris. The supernatant (39 ml) was applied to a DEAE-cellulose column (2.5 × 13.5 cm) equilibrated with buffer D. After washing the column with 120 ml of buffer D, the enzyme was eluted with a gradient of 0.1–1.0 mM NaCl of 270 ml. The active fractions were pooled (120 ml) and adjusted to 50% (NH4)2SO4. The precipitate was pelleted, redissolved to 10 ml, and desalted into buffer D using a 15-ml Swift desalting column (Pierce). The sample was applied to a Reactive Green 19-Agarose dye column (2.5 × 14 cm) equilibrated with buffer D. The column was washed with 120 ml of buffer D, and most of the enzyme was then eluted with a step gradient of 50 mM Tris-HCl, 0.5 mM NaCl, pH 7.5. The sample (25 ml) was adjusted to 50% (NH4)2SO4, and the precipitate was pelleted. The pellet was redissolved to 4.8 ml with buffer C, and then saturated (NH4)2SO4 (1.2 ml) was slowly added. The sample was centrifuged at 12,000 × g for 30 min to remove particulates before loading on the HIC column equilibrated with buffer B. The enzyme was eluted with a gradient of buffer C (1 ml/min, 1.2–0.6 mM (NH4)2SO4 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% (NH4)2SO4 for storage as a suspension at 4°C.

RESULTS AND DISCUSSION

The 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). The final step using reverse phase chromatography resolved three major peaks, which were sequenced by Edman degradation. The CMP-NeuAc synthetase was identified by homology to other known CMP-NeuAc synthetases.

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 and −80°C for at least several months. This material was used for initial characterization of the enzyme’s properties. The enzyme has a rather broad pH optimum from 8 to 9.5, which is similar to other CMP-NeuAc synthetases (30, 31). The enzyme has a requirement for Mg2+, with 20 mM giving the most activity under the assay conditions used. Mn2+ is only 40% as effective at 20 mM, whereas Ca2+ gave no reaction. The divalent cation requirements of other bacterial CMP-NeuAc synthetases are similar, whereas enzymes from animal sources are capable of using Ca2+ and other divalent cations in addition to Mg2+ and Mn2+ (30, 32). Sulphydryl reagents are necessary for or greatly stimulate the enzyme activity of some other CMP-NeuAc synthetases (30, 31). However, diithiothreitol was found to have little effect on enzyme activity and so was not included in purification.
tion buffers or enzyme assays. Interestingly, N-glycolylneuraminic acid was also a substrate for the H. ducreyi CMP-NeuAc synthetase. N-Glycolylneuraminic acid can be used as a substrate by CMP-NeuAc synthetases from animal tissues but not by the enzymes from E. coli or N. meningitidis (31). The monosaccharide, KDO, was not a substrate.

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 I). Approximately 1200 clones from a λDASHII library of strain 35000 DNA were screened with oligonucleotide 1, and seven 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 five clones had overlapping restriction maps. One clone was chosen for further characterization.

H. ducreyi DNA fragments of 5.5 and 8 kb were identified in a NotI digest from a λDASHII clone and subcloned into pWSK30. The restriction site designations are: N, NotI; B, BamHI; H, HindIII; and K, KpnI. The H. ducreyi neuA gene was localized on the NotI fragment by PCR analysis. The arrow designates the position and direction of transcription.

FIG. 2. Purification of the CMP-NeuAc synthetase from a H. ducreyi cell lysate. The fractions containing enzyme activity are shaded. A, anion exchange chromatography of active fractions from the size exclusion column. B, hydrophobic interaction chromatography of active fractions from A. C, rechromatography of active fractions from B on the HIC column using a shallower gradient. D, the fractions containing enzyme activity from C 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 an arrow) by its homology to other CMP-NeuAc synthetases.

FIG. 3. Partial restriction map of pRS1627. An 8-kb NotI fragment containing the H. ducreyi neuA gene was subcloned from a λDASHII clone into pWSK30. The restriction site designations are: N, NotI; B, BamHI; H, HindIII; and K, KpnI. The H. ducreyi neuA gene was localized on the NotI fragment by PCR analysis. The arrow designates the position and direction of transcription.

TABLE I
Oligonucleotides employed in this study

| Oligonucleotide | Sequence |
|-----------------|----------|
| 1               | 5' -ATGAARAAATGACGATATGAAACAA | |
| 2               | 5' -AARGGTATTCAGAAGGAAGAABAA | |
| 3               | 5' -AGATCATATGAAAGAGATTGCAATCATC | |
| 4               | 5' -CGGGATCCGGCAATTTCTTCATCGTAT | |

DNA fragments of 5.5 and 8 kb were identified in a NotI digest and cloned into NotI-digested pWSK30, and the mixture was transformed into E. coli DH5α. Clones containing either the 5.5- 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 on the NotI fragment by PCR analysis. The arrow designates the position and direction of transcription.

FIG. 4. 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 pRS1627. 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 1–43 correspond to the sequence determined by Edman degradation. Underlined nucleotides correspond to an inverted repeat characteristic of a rho-independent transcriptional terminator.
residue protein was identified (Fig. 4). 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 NdeI site (Table I, oligonucleotide 3), and the 3' primer contained a BamHI site (Table I, 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 H. ducreyi CMP-NeuAc synthetase. The gene was first amplified by PCR and recloned by TA cloning. The 5' primer contained an NdeI site (Table I, oligonucleotide 3), and the 3' primer contained a BamHI site (Table I, oligonucleotide 4). The sequence of the clone in the TA vector was again determined to verify that there were no PCR errors. The clone in the TA vector was identified 63 nucleotides downstream of the termination codon. Putative promoter sequences were not identified.

The enzyme was the major component of the soluble cell lysate (Fig. 5). 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. 6). A summary of the purification of the recombinant enzyme is given in Table II. The enzyme ran as a single band by SDS-PAGE with a molecular mass of 25.6 kDa. The enzyme also appeared homogeneous by reverse phase chromatography. The calculated average mass of 25440.6 Da was confirmed by electrospray mass spectrometry with an accuracy to within 1.4 Da (Fig. 7).

For CTP is much lower than values obtained for previous experiments (32). The E. coli enzyme is thought to be active as a monomer but did form dimers and larger aggregates in some buffers (47). The N. meningitidis enzyme also possibly exists as a dimer based on size exclusion chromatography results (29). 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. 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 Ref. 30 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, whereas the H. ducreyi enzyme was assayed at a more physiological pH.

There are now five sequences in the sequence data bases identified as CMP-NeuAc synthetases. A sixth sequence from Campylobacter coli is not identified as such but is highly similar (ptmB, U25992: identified as being involved in the post-translational modification of flagellin). All six sequences are

### Table II

| Chromatography step | Protein | Units | Specific activity | Purification | Recovery |
|---------------------|---------|-------|------------------|--------------|----------|
| Lysate              | mg      | µmol/min | µmol/min/mg | fold | % activity |
| 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        |
from bacteria. Twenty residues from the NH₂ 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 H. influenzae and N. meningitidis sequences being 65 and 45% identical (79 and 62% similar), respectively (Table III). These three enzymes all have nearly the same molecular weight, whereas the E. coli and Streptococcus agalactiae enzymes are nearly twice as large. The H. ducreyi CMP-NeuAc synthetase is 32% identical with the E. coli and S. agalactiae enzymes. 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 homology to the four known CMP-KDO synthetase sequences (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 (48–50). These proteins do not contain the HiGH motif that is involved in nucleotide binding in the recently elucidated cytidylyltransferase superfamily (51). The highest homology is found in the NH₂-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) (47). 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 MgCTP (Fig. 8). A conserved arginine and lysine are found nine residues apart in the NH₂ 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 (52, 53). In addition, there is almost a complete lack of negatively charged amino acids in this NH₂-terminal region. There is also a third region of high similarity with a conserved glutamine, preceded 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.

The NH₂-terminal regions of CMP-NeuAc and CMP-KDO synthetases also share some homology with other nucleotidyltransferases that catalyze the general reaction:

\[
\text{sugar-1-P + NTP} \leftrightarrow \text{NDP-sugar + PP}_i
\]

where NTP is a nucleotide triphosphate. The sequence alignment of several representative sugar nucleotidyltransferases is shown in Fig. 9. The conserved domains are aligned with a consensus sequence of ADP-glucose synthetases that is be-

### Table III

| Protein Accession Number | Protein | Accession Number | Protein Accession Number | Protein Accession Number |
|--------------------------|---------|-----------------|--------------------------|--------------------------|
| HD NeuA U54496           | HD NeuA U54496 | HD NeuA U54496 | HD NeuA U54496 | HD NeuA U54496 |
| HI SiaB L45913           | HI SiaB L45913 | HI SiaB L45913 | HI SiaB L45913 | HI SiaB L45913 |
| NM SynB X78068           | NM SynB X78068 | NM SynB X78068 | NM SynB X78068 | NM SynB X78068 |
| EC NeuA P13266           | EC NeuA P13266 | EC NeuA P13266 | EC NeuA P13266 | EC NeuA P13266 |
| SA CpsF U19899           | SA CpsF U19899 | SA CpsF U19899 | SA CpsF U19899 | SA CpsF U19899 |
| EC KdsB A26322           | EC KdsB A26322 | EC KdsB A26322 | EC KdsB A26322 | EC KdsB A26322 |
| HI KdsB L44702           | HI KdsB L44702 | HI KdsB L44702 | HI KdsB L44702 | HI KdsB L44702 |
| EC KpsU P42216           | EC KpsU P42216 | EC KpsU P42216 | EC KpsU P42216 | EC KpsU P42216 |
| CT KdsB U15192           | CT KdsB U15192 | CT KdsB U15192 | CT KdsB U15192 | CT KdsB U15192 |

a The abbreviations used are the same as in Fig. 8.
b The percentage of identical residues is given for each pair of proteins from the sequence alignment shown in Fig. 8. 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 homology in their carboxy-terminal region.
Fig. 8. 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 a high percentage of 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; and CT, Chlamydia trachomatis.

Fig. 9. 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.
H. ducreyi CMP-NeuAc Synthetase

lieved to be involved in phosphate binding (54). This domain, along with a second, were shown to be highly conserved in glucose-1-phosphate nucleotidyltransferases (55). 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 homology in the NH2 terminus, chemical modifications and site-directed mutagenesis experiments with the E. coli enzyme, and homology between CMP-NeuAc synthetases and other nucleotidyltransferases, it is quite possible that the NH2-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 recently reported that many strains contain terminal lactosamine and sialylated lactosamine as part of their LOS (12, 13). These epitopes are also a component of human blood group antigens and have important roles in molecular and cellular recognition (16, 18). Understanding the function of these sugars in the interaction of bacterial pathogens 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 (56-59), and LOS appears to play an important role in this process. Sialic acid may offer the bacteria more protection from the host immune defenses, but exposed terminal galactose may be necessary for the initial infection as has been suggested for other pathogens (60).

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.

Acknowledgments—We thank Lori Andrews for efforts in supplying the Edman sequence data and Prof. A. Campagnari (SUNY at Buffalo, NY) for efforts in supplying H. ducreyi CMP-NeuAc Synthetase. Based on the conserved positive charges and high sequence homology in the NH2 terminus, chemical modifications and site-directed mutagenesis experiments with the E. coli enzyme, and homology between CMP-NeuAc synthetases and other nucleotidyltransferases, it is quite possible that the NH2-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 recently reported that many strains contain terminal lactosamine and sialylated lactosamine as part of their LOS (12, 13). These epitopes are also a component of human blood group antigens and have important roles in molecular and cellular recognition (16, 18). 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 (56-59), and LOS appears to play an important role in this process. Sialic acid may offer the bacteria more protection from the host immune defenses, but exposed terminal galactose may be necessary for the initial infection as has been suggested for N. gonorrheae LOS (60). 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 galactosamine in adhesion to and invasion of host cells and susceptibility of the organism to host defenses. Such a mutant is currently under construction.

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.
Purification, Cloning, and Expression of a Cytidine 5′-Monophosphate N- Acetylneuraminic Acid Synthetase from Haemophilus ducreyi
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