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CMP-*N*-acetylneuraminic acid synthetase of *Escherichia coli*: high level expression, purification and use in the enzymatic synthesis of CMP-*N*-acetylneuraminic acid and CMP-neuraminic acid derivatives

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The gene encoding CMP-*N*-acetylneuraminic acid (CMP-NeuAc) synthetase (EC 2.7.7.43) in *Escherichia coli* serotype O7 K1 was isolated and overexpressed in *E.coli* W3110. Maximum expression of 8–10% of the soluble *E.coli* protein was achieved by placing the gene with an engineered 5′-terminus and Shine-Dalgarno sequence into a pKK223 vector derivative behind the *tac* promoter. The overexpressed synthetase was purified to >95% homogeneity in a single step by chromatography on high titre Orange A Matrex™ dye resin. Enzyme purified by this method was used directly for the synthesis of CMP-NeuAc and derivatives. The enzymatic synthesis of CMP-NeuAc was carried out on a multigram scale using equimolar CTP and *N*-acetylneuraminic acid as substrates. The resultant CMP-NeuAc, isolated as its disodium salt by ethanol precipitation, was prepared in an overall yield of 94% and was judged to be >95% pure by ^{1}H NMR analysis. *N*-Carbomethoxyneuraminic acid and *N*-carbobenzyloxyneuraminic acid were also found to be substrates of the enzyme; 5-azidoneuraminic acid was not a substrate of the enzyme. *N*-Carbomethoxyneuraminic acid was coupled to CMP at a rate similar to that observed with NeuAc, whereas *N*-carbobenzyloxyneuraminic acid was coupled >100-fold more slowly. The high level of expression achieved with the *E.coli* synthetase, together with the high degree of purity readily obtainable from crude cell extracts, make the recombinant bacterial enzyme the preferred catalyst for the enzymatic synthesis of CMP-*N*-acetylneuraminic acid.

Key words: CMP-*N*-acetylneuraminic acid/CMP-*N*-acetylneuraminic acid synthetase/enzymatic synthesis/sialylation

Introduction

Sialylated biomolecules are found widely in nature and have been implicated in a variety of biorecognition processes. The structures of naturally occurring sialylated species range from the polymeric forms of bacterial colominic acid to the various glycolipids and glycoproteins found in animals and man. Recent interest in sialylated biomolecules has been fuelled by studies on host cell recognition by a variety of mammalian pathogens including influenza A and B viruses (Suzuki *et al.*, 1986, 1987), Sendai virus (Suzuki *et al.*, 1983, 1984) and the bacterium *Mycoplasma pneumoniae* (Loomes *et al.*, 1984). The preparation of suitable substrates for the study of these host–pathogen interactive processes requires the isolation of naturally occurring sialylated biomolecules or, alternatively, the synthetic sialylation of glycoproteins and glycolipids.

The biosynthesis of sialylated biomolecules is dependent on two enzymatic reactions:

i) Sialic acid + CTP →CMP-sialic acid + PP_i
ii) CMP-sialic acid + glycosyl-OH→glycosyl-sialic acid + CMP

The first reaction, catalysed by CMP-*N*-acetylneuraminic acid (CMP-NeuAc) synthetase, activates NeuAc at the expense of one CTP equivalent. The second reaction, catalysed by a family of CMP-sialic acid glycosyltransferases, transfers sialic acid from the activated species to targeted glycosyl moieties. In similar fashion to the biological process, these two enzymes have been used for the synthetic preparation of a variety of sialylated glycoproteins (Rearick *et al.*, 1979; Higa and Paulson, 1985; Gross *et al.*, 1987; Gross and Brossmer, 1988) and glycolipids (Rearick *et al.*, 1979). The most common source of CMP-NeuAc synthetase used for enzymic CMP-NeuAc synthesis has been bovine brain. The enzyme prepared from this source, however, is relatively impure and contains a high level of phosphatase activity that significantly reduces the reaction yield (Higa and Paulson, 1985; Auge and Gautheron, 1988; Simon *et al.*, 1988).

It was of interest to determine if an overexpressed CMP-NeuAc synthetase, isolated from *Escherichia coli*, would provide a superior enzyme preparation for the enzymic synthesis of activated sialic acid derivatives. The CMP-NeuAc synthetase encoding gene from *E.coli* has been isolated (Vann *et al.*, 1987) and sequenced (Zapata *et al.*, 1989) previously. Using an oligonucleotide probe based on the preliminary sequence data of Vann *et al.* (1987), we have also obtained the synthetase encoding gene. This report describes the high level expression of CMP-NeuAc synthetase in *E.coli*, a convenient assay to monitor synthetase activity, a simple single-step column purification of the overexpressed enzyme and a protocol for use of the enzyme in the multigram scale synthesis of CMP-NeuAc.

Results and discussion

Overexpression of CMP-NeuAc synthetase

The gene encoding CMP-NeuAc synthetase of *E.coli* was isolated as described under Materials and methods. Initial expression experiments with the cloned CMP-NeuAc synthetase encoding gene were carried out by isolating the gene on a 2.7 kb *Eco*RI/*Hin*dIII fragment from pCMP.3 and ligating the fragment into the complementary sites on vector pKK223-NaeΔ behind the *tac* promoter (Figure 1). This

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construct, given the name pKKCMP.1, was used to transform *E.coli* W3110. Induction of the transformed cells with 2.0 mM isopropyl β-D-thiogalactoside (IPTG) yielded crude extracts with optimal CMP-NeuAc expression at ~1.8% of the soluble protein. To increase the yield of enzyme production, the synthetase encoding DNA insert was truncated at the 3′-terminus and both the 5′-terminus and Shine-Dalgarno sequences were engineered. Truncation was carried out by digesting the 2.7 kb *Eco*RI/*Hin*dIII fragment with *Nsi*I that cleaved 112 bp 3′-proximal to the synthetase termination codon. The resulting *Eco*RI/*Nsi*I synthetase encoding fragment, now ~1.4 kb in length, was ligated into the *Eco*RI/*Pst*I sites of vector pKK223-NaeΔ to yield vector pKKCMP.2. Induction of *E.coli* W3110 harbouring pKKCMP.2 showed an overall ~2-fold increase in synthetase expression level over that obtained with the pKKCMP.1 vector. In a final engineering experiment, the parent Shine-Dalgarno sequence was modified to yield a more extended ribosome binding site with a stretch of eight adenine residues 5′-proximal to the initiation codon. This extended ribosome binding site has been demonstrated to provide high efficiency in *E.coli* translational initiation (Sullivan *et al.*, 1989; Gold and Stromo, 1990). To carry out this manipulation, a single silent mutation was made in construct pKKCMP.2 to convert the unique *Xho*II site located 40 bp from the initiation codon to a unique *Bam*HI site. The synthetase Shine-Dalgarno sequence and 40 bp of the 5′-terminus were subsequently removed by digestion with *Eco*RI and *Bam*HI. The N-terminal encoding gene

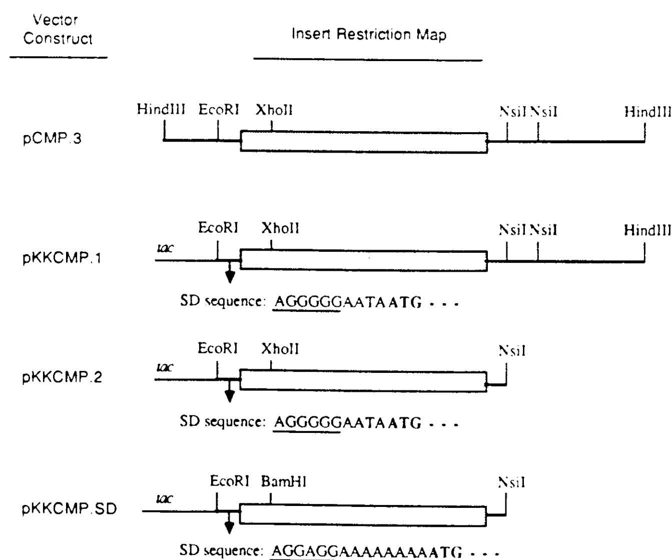

Fig. 1. Construction of CMP-NeuAc synthetase expression vectors. A graphical representation of the steps carried out to enhance the expression of the CMP-NeuAc synthetase encoding gene is shown. The *E.coli* CMP-NeuAc synthetase structural gene is represented by the stippled box and non-coding DNA as solid lines. The *tac* promoter lies 5″-proximal to the *Eco*RI site in vector pKK223.NaeΔ.

fragment with the desired Shine-Dalgarno sequence was then replaced by utilizing a 62 bp synthetic oligonucleotide cassette. In the course of this manipulation, several codons were modified to those expected in genes encoding highly expressed *E.coli* proteins (Gouy and Gautier, 1982). The sequence of the engineered ribosome binding site and 5′-terminus of the synthetase encoding gene is shown in Figure 2. This final construct, given the designation pKKCMP.SD, gave expression levels 5-fold higher than that achieved with vector pKKCMP.1 and resulted in typical specific activities of 0.25 U/mg in crude cell extracts of induced cells. This value is ca. 833-fold higher than that reported previously for expression of the isolated CMP-NeuAc synthetase encoding gene (Zapata *et al.*, 1989).

Purification of CMP-NeuAc synthetase

Recombinant CMP-NeuAc synthetase was purified ~11.2-fold from crude cell extracts of *E.coli* in an overall yield of 50–55%. Purification to >95% homogeneity, as judged by Coomassie blue staining of a 9.5% SDS–polyacrylamide gel (Figure 3), was routinely carried out by single-step column chromatography of crude cell extracts on high titre Orange A Matrex™ dye resin followed by ammonium sulphate fractionation and precipitation. The majority of contaminating proteins were removed in the chromatographic procedure by extensive washing of the Orange A Matrex™ dye resin. The CMP-NeuAc synthetase was purified further by elution from the dye resin with a linear KCl gradient. A typical protein elution profile from the Orange A Matrex™ dye column is shown in Figure 4. A slight increase in enzyme purity was subsequently achieved by fractionation of the pooled chromatographic fractions with 60% ammonium sulphate.

A specific activity of 2.5–2.8 U/mg was routinely obtained for the purified recombinant CMP-NeuAc synthetase. This value is comparable to the specific activity of 2.1 U/mg reported previously by Vann *et al.* (1987) for enzyme purified from a wild type *E.coli* strain. Minor contaminants remaining in the enzyme preparation may be removed by affinity chromatography on CDP-hexanolamine agarose (data not shown). However, for preparing enzyme to be used in the synthesis of CMP-NeuAc, this latter purification step was unnecessary.

The purification of overexpressed CMP-NeuAc synthetase from *E.coli* by chromatography on Orange A Matrex™ resin provides an efficacious method of enzyme recovery from crude cell extracts. Successful purifications were carried out when Orange A resin with a dye content of 3.77 and 4.22 mg/ml was employed. However, Orange A resin with 2.3 mg dye/ml only bound the synthetase weakly and was unsatisfactory for purification. The yield of purified synthetase of 10.7 U/g of *E.coli* W3110 transformed with vector pKKCMP.SD is >200-fold higher than that obtained from an equivalent quantity of bovine brain tissue.

M R T K I I A I I P A R S

Synthetase sequence: AATTCAAAAATCAGGGGGAATA ATG AGA ACA AAA ATT ATT GCG ATA ATT CCA GCC CGT AGT G - - -

Engineered sequence: AATTCATAAGGAGGAAAAAAAA ATG CGT ACT AAG ATC ATC GCC ATC ATC CCG GCC CGT AGT G - - -

Fig. 2. Sequence of the native and engineered ribosome binding sites and 5″-terminus of the CMP-NeuAc synthetase encoding gene. The Shine-Dalgarno sequences are underlined.

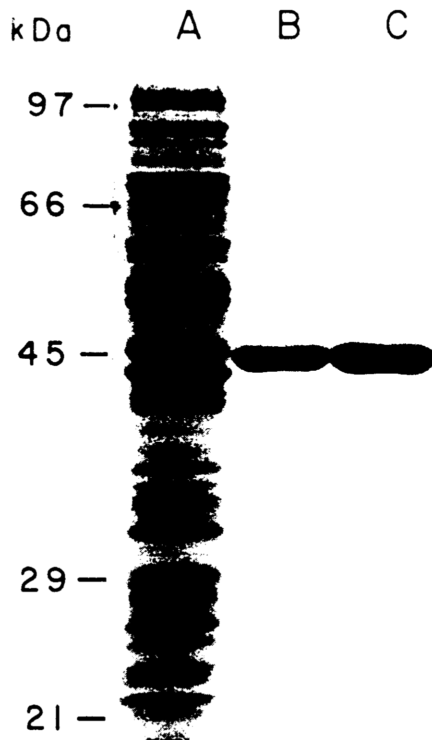

Fig. 3. Analysis of CMP-NeuAc synthetase purification by SDS–polyacrylamide gel electrophoresis. A 9.5 % polyacrylamide gel stained with Coomassie blue is shown. **(A)** 100 μg of crude cell extract; **(B)** 18 μg of purified CMP-NeuAc synthetase; **(C)** 30 μg of purified CMP-NeuAc synthetase.

Enzymatic synthesis of CMP-NeuAc

The multigram scale synthesis of CMP-NeuAc was carried out successfully in ~94% yield with the purified recombinant CMP-NeuAc synthetase as catalyst. The enzyme, as purified from *E.coli*, showed no detectable phosphatase or nucleotidase activity as evidenced by the stoichiometric conversion of CTP to the corresponding CMP-NeuAc derivative. This can be compared to syntheses that required the addition of 3 mole equivalents of CTP to drive the reaction to completion when the partially purified calf brain enzyme was used (Simon *et al.*, 1988). Furthermore, the high yield of purified synthetase readily obtained from 1 l of bacterial culture allows for the addition of more catalyst to the reaction mixture and, thus, decreases both the reaction time and the extent of spontaneous CTP hydrolysis.

The CMP-NeuAc obtained from the preparative synthetic reaction using the recombinant *E.coli* enzyme displayed ^{1}H (500 MHz) and ^{31}P (200 MHz) NMR spectral data identical to that obtained with authentic material from Sigma, as well as material prepared previously using the bovine brain enzyme (Simon *et al.*, 1988). Purity was judged to be >95% by the lack of additional resonances above the baseline of the high field spectra.

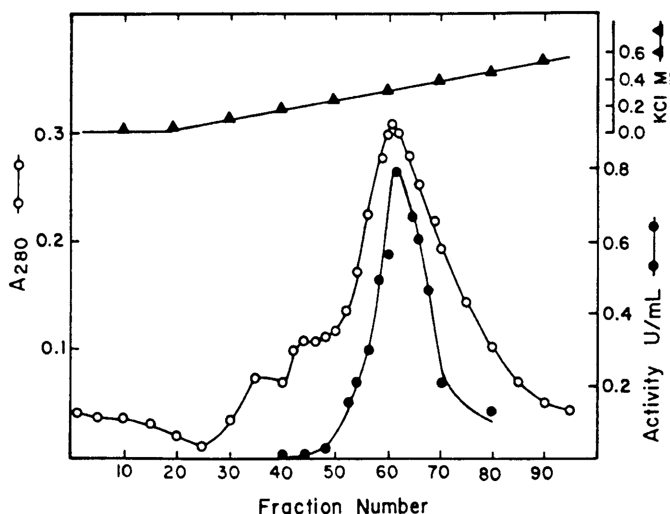

Fig. 4. Purification of CMP-NeuAc synthetase by chromatography on an Orange A Matrex™ dye column. A typical elution profile from an Orange A Matrex™ dye column with 4.22 mg dye/ml is shown. Activity was determined using a micro-scale assay method based on that described in Materials and methods.

Alternative substrates of CMP-NeuAc synthetase

To begin to examine the substrate specificity of *E.coli* CMP-NeuAc synthetase, three sialic acid derivatives, 5-*N*-carbobenzyloxy-, 5-*N*-carbomethoxy- and 5-azidoneuraminic acid were evaluated as CMP acceptors. As shown in Table I, the carbomethoxy and carbobenzyloxy derivatives of neuraminic acid were alternative substrates of the *E.coli* synthetase, whereas no reaction was detected with the 5-azido derivative. At a concentration of 5.0 mM, 5-*N*-carbomethoxyneuraminic acid was turned over at a rate equal to that observed with NeuAc and >100-fold faster than the rate observed with 5-*N*-Cbz-neuraminic acid. Because of limiting quantities of the NeuAc analogues, no further kinetic characterization was carried out. To determine if the enzymic synthesis of the CMP-NeuAc analogues could be carried out on a larger scale and to verify that the observed rates of P_i formation in the kinetic assays were indicative of CMP coupling, the synthetase reactions were carried out on a 0.1 mmol scale and monitored by ^{1}H NMR. Whereas the *N*-carbomethoxy derivative showed >80% product formation in 4 h and complete coupling in 14 h, the formation of CMP-*N*-Cbz-neuraminic acid only proceeded to ~10% conversion after 22 h. Thus, the relative rates observed in the preparative scale reactions were consistent with those found with the kinetic assay.

CMP-NeuAc synthetase of *E.coli* shows some tolerance for binding unnatural N-substituted neuraminic acid derivatives. The broad substrate specificity of the bovine enzyme for 9-substituted neuraminic acids has allowed for the enzymic synthesis of several novel CMP-NeuAc derivatives and glycoproteins (Gross *et al.*, 1987; Gross and Brossmer, 1988). Ultimately, a better understanding of the structural similarities between the bacterial and bovine enzymes may

Table I. Substrate specificity of CMP-NeuAc synthetase of *E.coli*

X[a]	Relative rate[b]	CMP adduct[c]
$H_3CC(O)NH$-	1	>90%, 4 h
$H_3COC(O)NH$-	1	>90%, 4 h
Cbz-NH-	<0.01	<10%, 22 h
N_3-	Not detectable	None detected

[a] Substitution at the C-5 position of sialic acid. Cbz = $C_6H_5CH_2OC(O)$-.
[b] Relative rate determined at 5.0 mM substrate under standard assay conditions.
[c] CMP adduct detected by ^{1}H NMR spectroscopy as described in the text. Values entered are estimates of percent conversion at given times.

evolve by examining similar 9-substituted sialic acid derivatives as substrates of the *E.coli* enzyme.

Conclusion

The enzymic synthesis of CMP-NeuAc from NeuAc and CTP is operationally simpler using recombinant CMP-NeuAc synthetase isolated from *E.coli* in comparison to using the enzyme isolated from calf brain for three reasons. First, the single-step isolation of overexpressed CMP-NeuAc synthetase from bacterial culture requires less effort than the multi-step procedure for isolating the enzyme from calf brain. Secondly, the absence of contaminating phosphatase activity in the *E.coli* preparation eliminates the requirement for excess CTP in the enzymic reaction mixture. Lastly, the absence of side products caused by contaminating enzyme activities in the calf brain preparation simplifies the isolation of CMP-NeuAc: a single ethanol precipitation step produces CMP-NeuAc of sufficient purity for use in subsequent enzyme-catalysed reactions.

Materials and methods

Inorganic pyrophosphatase and CTP were from Sigma. *N*-Acetylneuraminic acid was from either Sigma or Genzyme. Restriction enzymes and T4 DNA ligase were from Bethesda Research Laboratories. Calf intestine alkaline phosphatase was from Boehringer-Mannheim. Orange A Matrex™ dye resins were from Amicon. Sequenase DNA sequencing kit and IPTGwere from US Biochemical Corp. Sterox SE 0.02% was from VWR. γ-^{32}P-ATP and α-^{35}S-dATP were from Amersham. SDS–polyacrylamide gel electrophoresis molecular weight standards were from BioRad. All other biochemical reagents were the highest quality available from Sigma and Aldrich, unless otherwise indicated.

Neuraminic acid derivatives

The syntheses of 5-*N*-carbomethoxy-, 5-*N*-carbobenzyloxy- and 5-azidoneuraminic acids were carried out by Dr A.Schrell (Harvard University). Details of the syntheses are published elsewhere (Schrell and Whitesides, 1990).

Vectors and bacterial strains

E.coli serotype O7 K1 was obtained from the ATCC (strain £23503). *E.coli* W3110 was a gift from Dr N.Galakatos (CIBA-GEIGY Pharmaceuticals, Summit, NJ) and strain DH5α was from Bethesda Research Laboratories. All strains were grown at 37°C on 2 × TY medium that contained 16 g Bactotryptone, 10 g Bacto yeast extract and 5 g NaCl/l of culture medium. Growth medium was supplemented with 75 μg ampicillin/ml when required. For enzyme preparations, bacterial cultures were grown to an OD_{590} of 1.2–1.4 and induced by adjusting the medium to 2.0 mM IPTG. Cells were harvested by centrifugation following a 5.5-h induction period. Cloning vector pUC19 was from Bethesda Research Laboratories. Vector pKK223-NaeΔ was prepared by complete digestion of vector pKK223-3 (Pharmacia) with *Nae*I, followed by gel purification and ligation with T4 DNA ligase.

DNA manipulations and sequencing

All manipulations of DNA were carried out using standard techniques based on those described by Maniatis *et al.* (1982) or by manufacturers' instructions. Sequencing was performed by the dideoxy method of Sanger *et al.* (1977) according to the directions of the Sequenase kit. Oligonucleotides were prepared using an Applied Biosystems 380-B DNA synthesizer using standard phosphoramidite chemistry. The resultant oligonucleotides were purified by preparative polyacrylamide gel electrophoresis. When desired, oligonucleotides were phosphorylated on the 5′ end with T4 polynucleotide kinase prior to annealing. Oligonucleotides were annealed in 77 mM Tris buffer (pH 7.5), containing 11 mM $MgCl_2$ and 1 mM dithiothreitol (DTT) by heating to 65°C for 10 min, followed by slow cooling to room temperature.

Gene isolation

Genomic DNA from *E.coli* serotype O7 K1 was isolated by standard procedure and purified on a CsCl gradient (Maniatis *et al.*, 1982). The gene encoding CMP-NeuAc synthetase was isolated on a 3.7 kb *Hin*dIII fragment (Vann *et al.*, 1987) from a 2.5–4.4 kb size selected *Hin*dIII genomic DNA library constructed in pUC 19 vector. A total of 750 recombinants were screened with a ^{32}P-labelled oligonucleotide probe (ATGAGAACAAAAATTATTGCGATAATTCCAGCCCGTAGTGGA) that encodes the N-terminus of the synthetase (Vann *et al.*, 1987). Four positive clones were identified in the library and verified by restriction map and sequence analyses. One clone, designated pCMP-3, was in a preferred orientation and chosen for use in all further constructs. The identity of the isolated CMP-NeuAc synthetase clone was further confirmed by sequencing both the 5′ and 3′ regions of the isolated gene. The sequence obtained over these regions was identical to that reported by Zapata *et al.* (1989).

Enzyme assays

CMP-NeuAc synthetase activity was routinely assayed by coupling the synthetase reaction to inorganic pyrophosphatase and monitoring the production of inorganic phosphate with malachite green reagent (Lanzetta *et al.*, 1979). One unit (U) of activity is defined as the amount of enzyme required to produce 1 μmol of PP_i (2 μmol P_i) from CTP and NeuAc per min at 22°C. The standard assay mixture contained 0.1 M glycine (pH 9.5), 10 mM $MgCl_2$, 5.0 mM NeuAc, 1.5 mM CTP and 2 U of inorganic pyrophosphatase in 0.5 ml final volume. The reaction was initiated by the addition of 1–5 mU of CMP-NeuAc synthetase. Aliquots of 30 μl were removed at 5-min time intervals over 20–30 min and added directly to 0.8 ml malachite green reagent. After 1.5 min, the malachite green reaction was neutralized by the addition of 0.1 ml of 34% sodium citrate solution. The absorbance of the solutions was evaluated at 646 nm and the rate determined from the slope of the resulting line.

Protein concentrations were determined by using a microscale version of the biuret assay described by Layne (1957). Bovine serum albumin was used as a standard for the assay.

Purification of CMP-NeuAc synthetase

CMP-NeuAc synthetase from *E.coli* was routinely purified by chromatography on high titre Orange A Matrex™ dye resin followed by ammonium sulphate fractionation and precipitation. The purification buffer contained 50 mM Tris (pH 7.8), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT. All operations were carried out at 4°C. A description of a typical preparation follows.

The cell pellet obtained from 2 l of induced culture (~14 g frozen cell paste stored at −70°C) was resuspended in 50 ml buffer and the cells lysed by passage through a French pressure cell. Following brief sonication to shear genomic DNA (15 pulses, 2 s each), the cellular debri was removed by centrifugation. The resultant crude extract, with a CMP-NeuAc synthetase specific activity of 0.25 U/mg (360 U), was applied to a high titre (4.2 mg dye/ml) Orange A matrex™ dye column (2.5 × 30 cm) pre-equilibrated in purification buffer. After washing the column with 700 ml buffer, the remaining proteins were eluted with a 0.0–0.8 M KCl gradient prepared in purification buffer and 8.5 ml fractions collected. The fractions containing CMP-NeuAc synthetase activity (0.25–0.38 M KCl) were pooled and adjusted to 60% saturation with $(NH_4)_2SO_4$. The resulting precipitate was isolated by centrifugation, dissolved in 20 ml buffer and dialysed against 3 × 500 ml purification buffer. Particulates were removed by centrifugation and the CMP-NeuAc synthetase, with a specific activity of 2.7 U/mg, was precipitated by adjusting the dialysed sample to 60% saturation with $(NH_4)_2SO_4$. The $(NH_4)_2SO_4$ suspension of the synthetase was stored at 4°C for up to 6 months without detectable loss of activity.

Synthesis of CMP-NeuAc and analogues

All synthetic reactions were carried out in 50 mM Tris buffer (pH 8.5), containing 50 mM $MgCl_2$. The desired quantity of CMP-NeuAc synthetase for a given reaction was isolated from the $(NH_4)_2SO_4$ enzyme suspension by centrifugation. The resulting pellet was dissolved directly into the reaction mixture.

The preparative scale synthesis of CMP-NeuAc was carried out with 2.2 g CTP (3.1 mmol) and 1.0 g NeuAc (3.2 mmol) in 50 ml reaction buffer. The reaction was initiated by the addition of 8.0 U of *E.coli* CMP-NeuAc synthetase. The reaction was allowed to proceed for 3.5 h with stirring at

22°C and the pH was maintained at 8.5 by manual addition of 1 N NaOH. The resultant precipitate, presumably the magnesium-ammonium salt of pyrophosphate, was removed by filtration and washed with 25 ml distilled water. The filtrates were combined and the CMP-NeuAc precipitated by the addition of 675 ml ethanol (9:1 v/v). The CMP-NeuAc was isolated by centrifugation (10000 g, 10 min) and dried in vacuo at 0.1 torr to give 2.1 g of a white powder (2.9 mmol, 94%, yield assuming CMP-NeuAc·Na_2 of 95% purity).

Several NeuAc analogues were examined as substrates of the synthetase on a 0.1 mmol scale. Each analogue was dissolved in 3.0 ml of reaction buffer containing 0.1 mmol CTP and the reaction was initiated by the addition of 2.0 U of CMP-NeuAc synthetase. The progress of the coupling reactions was determined at 4, 14 and 22 h. Aliquots of 0.5 ml were removed from each reaction mixture at the appropriate time, frozen in liquid nitrogen and lyophilized. The resultant dry samples were dissolved in 0.5 ml D_2O and analysed by ^{1}H NMR (500 MHz). The diagnostic downfield shift of the sialyl C3-equatorial proton from δ 2.21 (dd) to 2.48 (dd) and the upfield shift and enhanced coupling pattern of the C3-axial proton from δ 1.85 (dd) to 1.65 (ddd), both characteristic of CMP-activated sialic acids, was monitored.

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Abbreviations

bp, base pair; DTT, dithiothreitol; IPTG, isopropyl β-D-thiogalactoside; kb, kilobase pair; NeuAc, *N*-acetylneuraminic acid; SDS, sodium dodecyl sulphate.

References

Auge,C. and Gautheron,C. (1988) An efficient synthesis of cytidine monophospho-sialic acids with four immobilized enzymes. *Tetrahedron Lett.*, **29**, 789–790.

Gold,L. and Stromo,G.D. (1990) High-level translation initiation. *Methods Enzymol.*, **185**, 89–93.

Gouy,M. and Gautier,C. (1982) Codon usage in bacteria: correlation with gene expressivity. *Nucleic Acids Res.*, **10**, 7055–7074.

Gross,H.J. and Brossmer,R. (1988) Enzymatic introduction of a fluorescent sialic acid into oligosaccharide chains of glycoproteins. *Eur. J. Biochem.*, **177**, 583–589.

Gross,H.J., Bunsch,A., Paulson,J.C. and Brossmer,R. (1987) *Eur. J. Biochem.*, **168**, 595–602.

Higa,H.H. and Paulson,J.C. (1985) Sialylation of glycoprotein oligosaccharides with N-acetyl, N-glycolyl, and N-O-diacetylneuraminic acids. *J. Biol. Chem.*, **260**, 8838–8849.

Lanzetta,P.A., Alvarez,L.J., Reinach,P.S. and Candia,O.A. (1979) An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.*, **100**, 95–97.

Layne,E. (1957) Spectrophotometric turbidimetric methods for measuring proteins. *Methods Enzymol.*, **3**, 447–454.

Loomes,L.M., Uemura,K., Childs,R.A., Paulson,J.C., Rogers,G.N., Scudder,P.R., Michalski,J-C., Hounsell,E.F., Taylor-Robinson,D. and Feizi,T. (1984) Erythrocyte receptors for *Mycoplasma pneumoniae* are sialylated oligosaccharides of Ii antigen type. *Nature*, **307**, 560–563.

Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Press, Cold Spring Harbor, NY.

Rearick,J.I., Sadler,J.E., Paulson,J.C. and Hill,R.L. (1979) Enzymatic characterization of β-D-galactoside α2-3 sialyltransferase from porcine submaxillary gland. *J. Biol. Chem.*, **254**, 4444–4451.

Sanger,F., Nicklen,S. and Coulson,A.R. (1977) DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.

Schrell,A. and Whitesides,G.M. (1990) Synthesis of the α-methyl ketoside of 5-aminoneuraminic acid methyl ester and its corresponding 5-myristoyl and 5-cyclopropanoyl derivative. *Liebigs Ann. Chem.*, 1111–1114.

Simon,E.S., Bednarski,M.D. and Whitesides,G.M. (1988) Synthesis of CMP-NeuAc from *N*-acetylglucosamine: generation of CTP from CMP using adenylate kinase. *J. Am. Chem. Soc.*, **110**, 7159–7163.

Sullivan,F.X., Shames,S.L. and Walsh,C.T. (1989) Expression of *Trypanosoma congolense* trypanothione reductase in *Escherichia coli*: overproduction, purification, and characterization. *Biochemistry*, **28**, 4986–4992.

Suzuki,T., Harada,M., Suzuki,Y. and Masumoto,M. (1984) Incorporation of sialoglycoprotein containing lacto-series oligosaccharides into chicken sialoerythrocyte membranes and restoration of receptor activity toward hemagglutinating virus of Japan (Sendai virus) *J. Biochem.*, **95**, 1193–1200.

Suzuki,Y., Suzuki,T. and Matsumoto,M. (1983) Isolation and characterization of receptor sialoglycoprotein for hemagglutinating virus of Japan (Sendai virus) from bovine erythrocyte membrane. *J. Biochem.*, **93**, 1621–1633.

Suzuki,Y., Nagao,Y., Kato,H., Matsumoto,M., Nerome,K., Nakajima,K. and Nobusawa,E. (1986) Human influenza A virus hemagglutinin distinguishes sialoligosaccharides in membrane-associated gangliosides as its receptor which mediates the adsorption and fusion processes of virus infection. *J. Biol. Chem.*, **261**, 17057–17061.

Suzuki,Y., Nagao,Y., Hideshige,K., Suzuki,T., Matsumoto,M. and Murayama,J. (1987) The hemagglutinins of the human influenza viruses A and B recognize different receptor microdomains. *Biochem. Biophys. Acta*, **903**, 417–424.

Vann,W.F., Silver,R.P., Abeijon,C., Chang,K., Aaronson,W., Sutton,A., Finn,C.W., Lindner,W. and Kotsatos,M. (1987) Purification, properties, and genetic location of *Escherichia coli* cytidine 5′-monophosphate *N*-acetylneuraminic acid synthetase. *J. Biol. Chem.*, **262**, 17556–17562.

Zapata,G., Vann,W.F., Aaronson,W., Lewis,M.S. and Moos,M. (1989) Sequence of the cloned *Escherichia coli* K1 CMP-*N*-acetylneuraminic acid synthetase gene. *J. Biol. Chem.*, **264**, 14769–14774.

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