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Kazuya ISHIGE[a], Tomoki HAMAMOTO[a], Toshikazu SHIBA[b] & Toshitada NOGUCHI[a]
[a] Biochemicals Division, YAMASA Corporation
[b] Division of Molecular Chemistry, Graduate School of Engineering, Hokkaido University
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Novel Method for Enzymatic Synthesis of CMP-NeuAc

Kazuya ISHIGE,† Tomoki HAMAMOTO, Toshikazu SHIBA,* and Toshitada NOGUCHI

Biochemicals Division, YAMASA Corporation, Choshi, Chiba 288-0056, Japan
**Division of Molecular Chemistry, Graduate School of Engineering, Hokkaido University, Sapporo 060-0810, Japan*

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A novel method for synthesizing CMP-NeuAc was established. We first confirmed that the putative *neuA* gene of *Haemophilus influenzae*, identified by its whole genome sequence project, indeed encodes CMP-NeuAc synthetase (EC 2.7.7.43). The enzyme requires CTP as a cytidylyl donor for cytidylylation of NeuAc. The enzyme was coupled with an enzymatic CTP-generating system from CMP and inorganic polyphosphate as a sole phospho-donor driven by the combination of polyphosphate kinase and CMP kinase, where phosphorylation of CMP is done by the combined activity expressed by both enzymes, and subsequent phosphorylation of CDP by polyphosphate kinase itself occurred efficiently. When CMP-NeuAc synthetase of *H. influenzae*, polyphosphate kinase, and CMP kinase were added to the reaction mixture containing equimolar concentrations (15 mM) of CMP and NeuAc, and polyphosphate (150 mM in terms of phosphate), CMP-NeuAc was synthesized up to 10 mM in 67% yield.

Key words: CMP-NeuAc; *Haemophilus influenzae*; inorganic polyphosphate; polyphosphate kinase; CMP kinase

Sialic acids are commonly observed in glycoconjugates like mammalian glycoproteins and glycolipids as the main terminal constituents, which extensively participate in cellular biorecognition processes. NeuAc has been demonstrated to play major roles in host cell recognition by a variety of mammalian pathogens including pathogenic bacteria and viruses and their toxins.[1,2] The biosynthesis of sialylated biomolecules is mediated by sialyltransferase using CMP-NeuAc, an activated form of NeuAc, as a sialic acid donor. For enzymatic preparation of sialylated biomolecules such as sialylorigosaccharides or gangliosides, there has been a great demand for CMP-NeuAc. Some approaches to produce CMP-NeuAc have been reported hitherto.[3–6] Because CMP-NeuAc synthetase (EC 2.7.7.43), responsible for biosynthesis of CMP-NeuAc, requires expensive CTP as a cytidylyl donor,[3] an enzymatic method to produce CMP-NeuAc should contain an appropriate CTP-generating system. Furthermore, CMP-NeuAc synthetase used for the production should be a bacterial one because of its easy preparation. The method described by Deie and Suguri[4] used yeast cells as a CTP-generating system from CMP and bovine tissue as an enzyme source for CMP-NeuAc synthetase, the latter of which is not readily available in a large quantity. Although Endo *et al.* have described a method using orotate and NeuAc as primary substrates[6] where *Corynebacterium ammoniagenes* cells convert orotate to UTP, *Escherichia coli* cells overproduce CTP synthetase catalyzing a conversion of UTP to CTP, and cells that overproduce CMP-NeuAc synthetase are coupled, it requires tremendous doses of the bacterial cells.

Biosynthesis of nucleoside triphosphates (NTPs) from cognate nucleoside monophosphates (NMPs) are mediated by several enzymes. ATP-dependent phosphorylation of NMPs to give cognate nucleoside diphosphates (NDPs) are catalyzed by nucleoside monophosphate kinases (NMPKs) such as adenylate kinase, GMP kinase, CMP kinase, and UMP kinase, which phosphorylate each corresponding NMP.[7–10] Subsequent phosphorylation of NDPs to cognate NTPs are mainly catalyzed by nucleoside diphosphate kinase (NDK) which has a broad specificity for phospho-acceptors to phosphorylate all four NDPs with ATP,[11] and ADK has been reported to have an NDK-like activity.[12] Although NMPs are inexpensively and abundantly available, an NTP-generating system using the enzymes described above requires expensive ATP. The coupling of an ATP-regenerating system driven by creatine kinase or pyruvate kinase can reduce the dose of ATP, which in turn requires still expensive phosphocreatine or phosphoenolpyruvate (PEP) as

† To whom correspondence should be addressed. Tel: 81-479-22-0095; Fax: 81-479-22-9865; E-mail: ishige-k@yamasa.com

Abbreviations: NDK, nucleoside diphosphate kinase; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; NMPK, nucleoside monophosphate kinase; NTP, nucleoside triphosphate; PEP, phosphoenolpyruvate; polyP, inorganic polyphosphate; PPK, polyphosphate kinase

an alternative phospho-donor, respectively.

Among the four NTPs, ATP or CTP can be synthesized from cognate NMPs also in an inorganic polyphosphate (polyP)-dependent manner. It has been known that inorganic polyphosphate kinase (PPK) responsible for the processive synthesis of polyP from ATP in *E. coli*[13-15] catalyzes polyP-dependent phosphorylation of all four NDPs.[16] Furthermore, we previously demonstrated that polyP and PPK function as an ATP substitutes for adenylate kinase[17] or CMP kinase (unpublished results) to give polyP:AMP or :CMP phosphotransferase activities. PolyP is the cheap material which can serve as a phospho-donor for phosphorylation of nucleotides. A combination of PPK and each NMPK might be the most plausible enzymatic NTP-generating system using polyP as a sole phospho-donor. Here, we describe a novel method for enzymatic synthesis of CMP-NeuAc using the CMP-NeuAc synthetase of *H. influenzae* characterized in this study and the combination of PPK and CMP kinase as the efficient CTP-generating system.

Materials and Methods

Plasmids and strains. The putative *neuA* gene[18] of *H. influenzae* (ATCC9745) was amplified by PCR with primers 5′-TGCCATGGTGAAAATAATAATGACAAGAA-3′ and 5′-AACTGCAGTGCAGATCAAAAGTGCGGCC-3′ and the resulting PCR product was cloned into the *Nco*I-*Pst*I site of pTrc99A[19] to give pTrc-siaBNP harboring the *neuA* gene, which has an additive start codon ATG. The *E. coli cmk* gene[20] encoding CMP kinase was amplified by PCR with primers 5′-TACCATGGAGATAAAGATGACGGCAATT-3′ and 5′-ATGGATCCTGCAAATTCGGTCGCTTATGC-3′ and the resulting PCR product was cloned into *Nco*I-*Bam*HI site of pTrc99A to give pTrc-CMK. Both genes were placed under the control of the *trc* promoter. *E. coli* JM109[21] was used as the host cell for overexpression of the genes.

Overexpression of H. influenzae neuA gene in E. coli. E. coli JM109 harboring pTrc-siaBNP was cultured in 2×YT[22] broth with 50 μg/ml of ampicillin at 30°C until the mid-logarithmic growth phase. Then IPTG was added to 1 mM and cultivation was continued for 5 h. Five grams of each of the harvested cells was suspended in 50 ml of TED buffer (50 mM Tris-HCl (pH 8.0), 0.5 mM EDTA and 1 mM 2-mercaptoethanol). The cell suspension was sonicated and centrifuged at 30,000×*g* for 30 min to prepare the cell lysate.

Enzyme preparation for CMP-NeuAc production. Overproduction followed by purification of PPK was done as described previously.[17] Cultivation of *E. coli* JM109 harboring pTrc-CMK followed by preparation of the cell lysate was done as described in the former section. CMP kinase was purified from the cell lysate as follows. Solid ammonium sulfate was added to the cell lysate containing the overproduced enzyme to 45% saturation. The precipitates formed were recovered and dissolved in 10 ml of TED buffer. The sample was put onto a Sephacryl S-300 HR column (2.2×32 cm; Amersham Pharmacia) equilibrated with TED buffer, and separated by isocratic elution with TED buffer. The CMP kinase-fractions were then put on a DEAE-Toyopearl 650M column (2.2×32 cm; Toso, Tokyo, Japan) equilibrated with TED buffer, followed by elution with 1 L of linear NaCl gradient from 0 to 500 mM to give homogeneous CMP kinase. CMP-NeuAc synthetase was purified from the lysate of *E. coli* JM109 harboring pTrc-siaBNP as follows. Solid ammonium sulfate was added to the cell lysate (50 ml) of the overproducer to 45% saturation. The precipitates formed were recovered and dissolved in 10 ml of TED buffer. The sample was put onto a DEAE-Toyopearl 650S column (2.2×32 cm; Toso, Tokyo, Japan) equilibrated with TED buffer, followed by elution with 1 L of linear gradient of NaCl from 0 to 500 mM. The CMP-NeuAc synthetase-fractions were put on an AKTA FPLC column system with ResourceQ (1 ml) column (Amersham Pharmacia) followed by elution with 20 ml of linear NaCl gradient from 0 to 500 mM. The resultant CMP-NeuAc synthetase-fractions were then put on an AKTA FPLC column system with HiLoad 16/60 Surperdex 200 pg column (Amersham Pharmacia) equilibrated with TED buffer, and separated by isocratic elution with TED buffer to give the homogeneous enzyme. The enzymes were monitored by SDS-PAGE.[23]

Enzyme assays. The PPK assay mixture contained 50 mM Tris-HCl (pH 8.0), 50 mM ammonium sulfate, 10 mM $MgCl_2$, 5 mM ADP, polyP (150 mM in terms of phosphate) and an enzyme sample. The CMP kinase assay mixture contained 50 mM Tris-HCl (pH 8.0), 50 mM ammonium sulfate, 10 mM $MgCl_2$, 10 mM ATP, 5 mM CMP and an enzyme sample. The CMP-NeuAc synthetase assay mixture contained 100 mM Tris-HCl (pH 8.0), 20 mM $MgCl_2$, 5 mM CTP, 5 mM NeuAc and an enzyme sample. The products were measured by HPLC. One unit of an enzyme was defined as the amount required to form 1 μmol of ATP in the PPK assay in a polyP-dependent manner, 1 μmol of CDP in the CMP kinase assay, and 1 μmol of CMP-NeuAc in the CMP-NeuAc synthetase assay per min at 30°C.

Recombinant DNA techniques. Chromosomal DNA of *H. influenzae* (ATCC9745) was obtained from Mr. N. Matsumoto. DNA-manipulating enzymes, such as restriction endonucleases and T4

DNA ligase, were used under the recommended conditions by the suppliers (Takara Shuzo, Kyoto, Japan). Other recombinant DNA techniques were done as described by a laboratory manual.[22]

Chemicals and other supplies. PolyP (chain lengths, 13–18) and inorganic pyrophosphatase used in this study were purchased from Sigma. CMP was prepared by Yamasa Corporation (Chiba, Japan). NeuAc and the other chemicals were obtained from Nacalai tesque (Kyoto, Japan).

Results

Preparation of H. influenzae CMP-NeuAc synthetase

CMP-NeuAc synthetase (EC 2.7.7.43) is the key enzyme to synthesize CMP-NeuAc, which catalyzes cytidylylation of NeuAc using CTP as a cytidylyl donor.[3] The existence of the enzyme in *H. influenzae* has been inferred from its genome sequence. We focused on the putative *neuA* gene[18] of *H. influenzae* identified by the whole genome sequence project, the ORF of which is composed of 687 bp encoding a protein of 228 amino acids with a calculated mass of 25308 Da. The deduced amino acid sequence of the gene product shows extensive similarity to those of known CMP-NeuAc synthetases. Among them, it has 65.8% identity to the enzyme of *H. ducreyi*[24] in the amino acid sequence (data not shown). Although *E. coli* JM109 has no detectable CMP-NeuAc synthetase activity, overexpression of the *neuA* gene in the strain resulted in a massive expression of the activity (Table 1), confirming that the gene indeed encodes CMP-NeuAc synthetase. The intrinsic start codon of the gene is GTG which is the inefficient one for translational initiation in *E. coli*. To improve the productivity of the enzyme we added the usual start codon ATG to the coding sequence, replacing the intrinsic N-terminal amino acid residue Met with Met-Val. The replacement of the native codon resulted in a 3-fold increase in productivity of the enzyme (data not shown).

The enzyme was purified to homogeneity from the lysate of the overproducers for use in synthesizing CMP-NeuAc. The specific activity of the purified enzyme was 6.25 units/mg protein, which is 2.3-fold higher than that of the pathogenic *E. coli* one previously reported.[3]

Table 1. CMP-NeuAc Synthtase Activity of the Lysate of the Overproducers

Lysate	Specific activity units/mg proteins
Control[a]	<0.01
neuA[+][b]	3.40

[a] The lysate of *E. coli* JM109 harboring control vector pTrc99A.
[b] The lysate of *E. coli* JM109 harboring pTrc-siaBNP.

Enzymatic synthesis of CTP from CMP using polyP as a sole phospho-donor

It had been suggested that the combination of PPK and CMP kinase could be used for an enzymatic CTP-generating system from CMP using polyP as a sole phospho-donor in which both enzymes synergistically phosphorylate CMP with polyP and the resulting CDP is further phosphorylated with polyP by PPK itself. Each of PPK and CMP kinase was overproduced in *E. coli*, and then purified to homogeneity. The specific activity of both enzymes were 0.503 units/mg protein for PPK, and 37.0 units/mg protein for CMP kinase. The combined activity of PPK and CMP kinase to phosphorylate CMP with polyP is affected by the molar ratio of both enzymes themselves (our unpublished results). For synthesis of CTP from CMP and polyP, PPK and CMP kinase of 5 times the molar amount were used. When both enzymes were incubated with CMP and polyP, sequential phosphorylations of CMP were observed. After 16 h of incubation, CMP was converted to CTP in 50% yield (Fig. 1), confirming that phosphorylation of CMP by CMP kinase using polyP and PPK as an ATP-substitute, and subsequent phosphorylation of CDP by polyP-dependent NDK activity of PPK itself are efficiently coupled. When polyP was omitted from the reaction mixture, production of neither CDP nor CTP was observed (data not shown), indicating that the reactions are

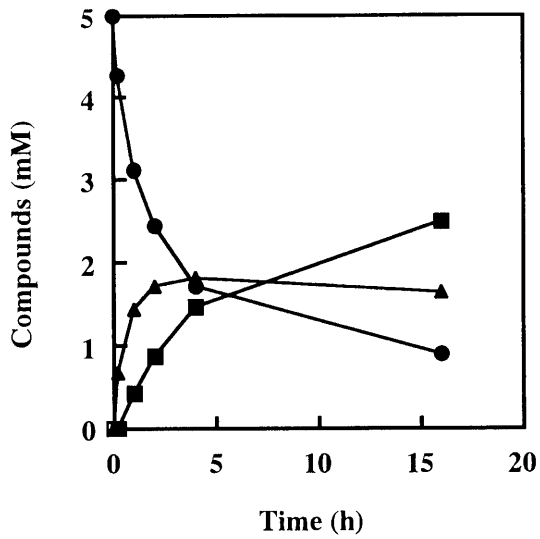

Fig. 1. The Course of the Enzymatic Synthesis of CTP from CMP and PolyP.

The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 50 mM ammonium sulfate, 20 mM $MgCl_2$, 5 mM CMP, polyP (75 mM in terms of phosphate), 0.2 nM (0.08 units/ml) PPK, and 1 nM (10 units/ml) CMP kinase. The mixture was incubated at 37°C. The reaction was stopped by boiling. The compounds were analyzed by HPLC. Symbols: circles, CMP; triangles, CDP; squares, CTP.

completely dependent on polyP.

Enzymatic synthesis of CMP-NeuAc from CMP, NeuAc, and polyP

The scheme for synthesizing CMP-NeuAc is shown in Fig. 2. The sequential phosphorylations of CMP with polyP to give CTP by the combination of PPK and CMP kinase, and subsequent cytidylylation of NeuAc to synthesize CMP-NeuAc by CMP-NeuAc synthetase should occur simultaneously in a single reaction vessel. To prevent the inhibition of CMP-NeuAc synthetase from a by-product pyrophosphate, inorganic pyrophosphatase was added to the reaction mixture. When equimolar concentrations (15 mM) of CMP and NeuAc, and polyP (150 mM in terms of phosphate) as a phospho-donor for sequential phosphorylations of CMP were added to the reaction mixture, the enzymes mediated the synthesis of CMP-NeuAc up to 10 mM in 67% yield (Fig. 3). Note that the reaction yield is higher than that of the CTP generating system alone driven by the combination of PPK and CMP kinase (see Fig. 1). We speculate that keeping the CTP level low by continuous synthesis of CMP-NeuAc might release PPK and/or CMP kinase from the product inhibition.

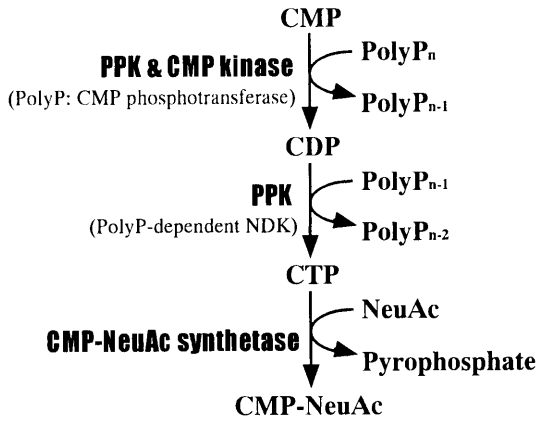

Fig. 2. The Scheme for the Enzymatic Synthesis of CMP-NeuAc.

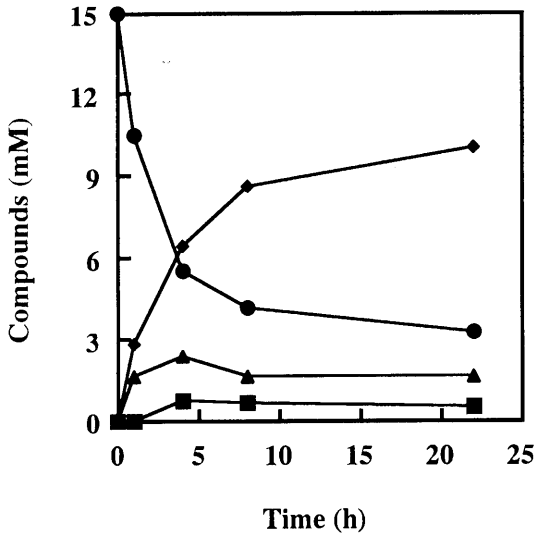

Fig. 3. The Course for the Enzymatic Synthesis of CMP-NeuAc. The reaction mixture contained 100 mM Tris-HCl (pH 8.0), 50 mM ammonium sulfate, 50 mM $MgCl_2$, 15 mM CMP, 15 mM NeuAc, polyP (150 mM in terms of phosphate), 1.0 units/ml of pyrophosphatase, 0.2 nM (0.08 units/ml) of PPK, 1 nM (10 units/ml) of CMP kinase, and 0.2 units/ml of CMP-NeuAc synthetase. The mixture was incubated at 37°C. The reaction was stopped by the addition of ethanol up to 35%. The compounds were analyzed by HPLC. Symbols: circles, CMP; triangles, CDP; squares, CTP; diamonds, CMP-NeuAc.

Discussion

We first confirmed that the putative *neuA* gene of *H. influenzae* indeed encodes CMP-NeuAc synthetase, the amino acid sequence of which is most similar to that of *H. ducreyi* of known CMP-NeuAc synthetases. The specific activity of the purified enzyme was 2.3-fold higher than that of *E. coli* K1's one, indicating that the *H. influenzae* enzyme is suitable for practical use. Although purified enzymes were used in this study in order to demonstrate that using only the enzymes described here CMP-NeuAc can be synthesized from CMP and NeuAc using polyP as a sole phospho-donor, we have already confirmed that highly purified enzymes are not needed for CMP-NeuAc synthesis; leading to a reduction of the manufacturing cost (data not shown).

It was confirmed that the combination of PPK and CMP kinase efficiently produce CTP from CMP using polyP as a sole phospho-donor, indicating that phosphorylation of CMP by CMP kinase using polyP and PPK as an ATP-substitute, and subsequent phosphorylation of CDP with polyP by polyP-dependent NDK activity of PPK itself are efficiently coupled. In coupling with *H. influenzae* CMP-NeuAc synthetase, this CTP-generating system produced a novel method for enzymatic synthesis of CMP-NeuAc from CMP and NeuAc, which does not require expensive phospho-donors such as ATP or PEP. PolyP is an extremely low-priced material which can serve as a phospho-donor for phosphorylation of nucleotides compared to ATP or PEP, suggesting that the method described here is a plausible way to synthesize CMP-NeuAc economically.

PolyP serves as the donor not only for the sequential phosphorylations of CMP to CTP described here, but also for AMP-phosphorylation by adenylate kinase in combination with PPK,[17] and the further phosphorylation to ATP by PPK itself. We have already established the efficient ATP-generation system from AMP driven by the combination of PPK and adenylate kinase using polyP as a sole phospho-donor (data not shown). We are currently trying to apply the enzymatic CTP- or ATP-generating system to synthesis of other nucleotide derivatives.

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