ENZYMATIC FORMATION OF URIDINE DIPHOSPHATE *N*-ACETYL-D-MANNOSAMINURONIC ACID

Naoshi ICHIHARA, Nobutoshi ISHIMOTO and Eiji ITO
Department of Chemistry, Faculty of Science, University of Hokkaido, Sapporo, Japan

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1. Introduction

Several hexosaminuronic acids have been discovered in a number of microorganisms. Mannosaminuronic acid was isolated from K7 specific polysaccharide of *Escherichia coli* [1], the polysaccharide of *Micrococcus lysodeikticus* cell wall [2] and the specific polysaccharide of *Staphylococcus aureus* [3]. In the studies of the biosynthesis of mannosaminuronic acid-containing polysaccharide, the occurence of UDP-GlcNAcUA [4, 5] and UDP-ManNAcUa [5] in *M. lysodeikticus* and the utilization of the latter nucleotide as substrate in the enzymatic synthesis of its cell wall polysaccharide [6] have been reported, but the mechanism of synthesis of these nucleotides is unknown.

We report here that the incubation of UDP-GlcNAc with a soluble enzyme of *E. coli* 014 K7 H^- in the presence of NAD^+ resulted in formation of UDP-HexNAcUA which was identified chemically and enzymatically as UDP-ManNAcUA.*

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Abbreviations: UDP–ManNAcUA, UDP-*N*-acetyl-D-mannosaminuronic acid; UDP-GlcNAcUA, UDP-*N*-acetyl-D-glucosaminuronic acid; UDP-HexNAcUA, UDP-*N*-acetylhexosaminuronic acid; UDP-GlcNAc, UDP-*N*-acetyl-D-glucosamine; ManNAc, *N*-acetyl-D-mannosamine.

2. Materials and methods

E. coli 014 K7 H^-, furnished by Drs. I. and F. Ørskov, Statens Serum Institute, Copenhagen, was grown in the Brain Heart Infusion Medium (Difco). *E. coli* cells from 2 liters of culture were harvested at 60% maximal growth, and suspended in 20 ml of 0.03 M Tris–HCl, pH 7.6. The suspension was treated in a 10 Kc sonic oscillator for 3 min. The homogenate was centrifuged at 20 000 *g* for 20 min and the supernatant was further centrifuged at 100 000 *g* for 60 min. The supernatant was used as the soluble enzyme. The cells of *M. lysodeikticus* ATCC 4698 were grown in a medium containing 2% peptone, 0.5% yeast extract and 0.5% NaCl. The particulate enzyme and the heated supernatant fraction from *M. lysodeikticus* were prepared by the procedure described by Page et al. [6].

Glucosaminuronic acid was prepared by hydrolysis in 1 N HCl for 1 hr at 100°C of synthetic GlcNAcUA-lactone which was a gift from Dr. H. Ando, Tokyo Technical University.

Mannosaminuronic acid was prepared as described by Biely et al. [4] except that ECTEOLA-cellulose column chromatography in place of cetylpyridinum chloride precipitation was used.

Glucosamine labeled UDP-[^{14}C]GlcNAc was prepared as described by Glaser et al. [7]. UDP-[^{14}C]glucose was purchased from RCC, England. NAD^+ and UDP-GlcNAc was purchased from Boehringer.

The reduction of UDP-[^{14}C]HexNAcUA to UDP-[^{14}C]HexNAc was carried out as described by Biely et al. [4].

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Isolation of enzymatically synthesized UDP-ManNAcUA was carried out as follows. 0.1 mmole of UDP-GlcNAc were incubated at 37°C for 1 hr in a mixture containing 5 mmoles of Tris–HCl buffer, pH 8.6, 0.2 mmole of NAD^+, and 40 ml of the soluble enzyme in a total volume of 100 ml. The reaction was stopped by boiling the incubation mixture for 5 min. After removal of the protein precipitate by centrifugation, the supernatant was chromatographed on Toyo No. 50 filter paper in isobutyric acid–0.5 M NH_4OH (5:3) (solvent 1). The ultraviolet-absorbing and slowest moving band was extracted from the paper with water, and the solution was further chromatographed on paper in ethanol-1 M ammonium acetate pH 7.3 (75:30) (solvent 2). The band, having $R_{UDP\text{-}GlcNAc}$ 0.68, of material corresponding to UDP-HexNAcUA, was extracted with water and further purified in solvent 1 and solvent 2. Fifty μmoles of UDP-HexNAcUA was obtained. Hexosamine labeled UDP-[^{14}C] HexNAcUA was also obtained from glucosamine labeled UDP-[^{14}C] GlcNAc.

3. Results and discussion

Incubation of UDP-[^{14}C] GlcNAc with the soluble enzyme of *E. coli* resulted in formation of UDP-[^{14}C] HexNAcUA as shown in table 1. The addition of NAD^+ was necessary for the maximum formation of UDP-HexNAcUA, suggesting that the reaction is analogous to the NAD^+-dependent reaction by UDP-glucose dehydrogenase or UDP-glucuronic acid-4-epimerase in *Streptococcus pneumoniae* described by Smith et al. [8]. Although the soluble enzyme could be stored frozen at – 20°C for several weeks without a significant loss of activity, it was very unstable on chromatography in Sephadex or DEAE-cellulose, and therefore, the purification of this enzyme was difficult.

Identification of UDP-HexNAcUA was carried out as follows. It had the typical spectra of uridine at pH 7 and 12. On analysis it gave ratios of uridine: total phosphate: reducing sugar [9] : acetylamino sugar [10] of 1.00:1.95:0;73:0.58. GlcNAc was used as a standard in the reducing sugar and *N*-acetylamino sugar determination. After acid hydrolysis of UDP-HexNAcUA in 1 N HCl for 1 hr at 100°C, the resulting carbohydrate component was purified by chromatography using pyridine-n-butanol-CH_3COOH-H_2O (40:60:3:30). As shown in table 2, the isolated component was identical with authentic mannosaminuronic acid in its behavior on chromatography and electrophoresis. It also showed the characteristic ninhydrin staining of hexosaminuronic acid [2].

After mild hydrolysis of reduced UDP-[^{14}] HexNAcUA in 0.01 N HCl for 15 min at 100°C, the resulting

Table 1
Formation of UDP-HexNAcUA

Assay conditions	UDP-HexNAcUA formation
	nmoles
Complete	0.86
– NAD^+	0.24
Boiled soluble enzyme	0.00

The reaction mixtures, in a final volume of 50 μl, contained UDP-[^{14}C] GlcNAc (28 000 cpm, 2.8 nmoles), 50 mM Tris–HCl, pH 8.6, 1.2 mM NAD^+ and the soluble enzyme (400 μg of protein). After incubation for 45 min at 37°C, the reaction mixtures were subjected to chromatography in solvent 2 for 24 hr. The UDP-HexNAcUA area ($R_{UDP\text{-}GlcNAc}$ 0.68) was counted.

Table 2
Mobilities in paper chromatography and paper electrophoresis

Samples	Solvent 3	Solvent 4	Buffer 1
Hexosaminuronic acid synthesized enzymatically	1.12	1.16	1.19
Mannosaminuronic acid	1.12	1.17	1.19
Glucosaminuronic acid	1.00	1.00	1.00
Glucosamine	3.06	3.77	0.00

Descending paper chromatography and paper electrophoresis were carried out on Toyo No. 50 filter paper. Solvent 3, pyridine-n-butanol–CH_3COOH–H_2O (60:40:3:30); Solvent 4, n-pentanol–pyridine–H_2O (7:7:6); Buffer 1, 0.1 M sodium molybdate, pH 5.0, at 13 V/cm for 90 min. The spots were detected with the silver nitrate reagent.

Table 3
Utilization of UDP-HexNAcUA for *M. lysodeikticus* polysaccharide biosynthesis

Assay conditions	Incorporation of glucose
	nmoles
Complete	0.56
– UDP-HexNAcUA	0.02
– UDP-GlcNAc	0.09
– $MgCl_2$	0.17
– Particulate enzyme	0.02

The reaction mixtures, in a final volume of 22 μl, contained 0.21 mM UDP-[^{14}C]Glc (1,200 cpm/nmole), 0.4 mM UDP-HexNAcUA, 0.4 mM UDP-GlcNAc, 50 mM HEPES buffer, pH 8.5, 20 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 0.2 vol of the heated supernatant fraction and the particulate enzyme fraction (96 μg of protein). The mixtures were incubated for 120 min at 37°C and the radioactivity incorporated into a polysaccharide was determined [6].

N-acetylamino sugar moiety had the same mobility as ManNAc on paper chromatography using the borate (15 mM of tetraborate, pH 10.0)-soaked paper in pyridine-n-butanol-H_2O (4:6:3).

In addition, by the capability of UDP-HexNAcUA to serve as substrate in *M. lysodeikticus* polysaccharide synthesis, it was further supported that the enzymatically synthesized UDP-HexNAcUA was UDP-ManNAcUA. It has been reported that in vitro incorporation of D-glucose from UDP-D-glucose into wall polysaccharide by the particulate enzyme from *M. lysodeikticus* is dependent upon UDP-ManNAcUA and UDP-GlcNAc [5]. As shown in table 3, addition of UDP-HexNAcUA was necessary for the incorporation of D-glucose from UDP-D-glucose in the assay system of polysaccharide synthesis.

The present results clearly indicate the enzymatic formation of UDP-ManNAcUA from UDP-GlcNAc. Therefore, the biosynthesis of UDP-ManNAcUA in *E. coli* would occur through epimerization and oxidation at the level of nucleotide-sugar, but not at the level of sugar, sugar-phosphate or polysaccharide, although the mechanism of the epimerization and the oxidation of UDP-GlcNAc remains to be studied.

References

[1] Mayer, H., (1969) Eur. J. Biochem. 8, 139.
[2] Perkins, H.R., (1963) Biochem. J. 86, 475.
[3] Wu, T.C.M. and Park, J.T. (1971) J. Bacteriol. 108, 874.
[4] Biely, P. and Jeanloz, R.W. (1969) J. Biol. Chem. 244, 4929.
[5] Anderson, J.S., Page, R.L. and Salo, L. (1972) J. Biol. Chem. 247, 2480.
[6] Page, R.L. and Anderson, J.S. (1972) J. Biol. Chem. 247, 2471.
[7] Glaser, L. and Brown, D.S. (1955) Proc. Natl. Acad. Sci. U.S. 41, 253.
[8] Smith, E.E.B., Mills, G.T., Austrian, R. and Berhneimer, H.P., (1960) J. Gen. Microbiol. 22, 265.
[9] Park, J.T. and Johnson, M. (1949) J. Biol. Chem. 181, 149.
[10] Reissig, J.L., Strominger, J.L. and Leloir, L.F. (1955) J. Biol. Chem. 217, 959.