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Characterization of a key aminoglycoside phosphotransferase in gentamicin biosynthesis

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ABSTRACT

Gentamicin is an aminoglycoside antibiotic obtained from cultures of *Micromonospora* as the important anti-infective agents. Gentamicin which lacks 3′-hydroxyl group can avoid the attack from the modification enzymes of antibiotic-resistant bacteria in clinic. Consequently, C-3′ dehydroxylation is the key step in gentamicins biosynthesis. We suppose that there are some enzymes responsible for converting intermediate JI-20A to 3′,4′-bisdehydroxylated final product gentamicin C_{1a}, while phosphorylation of 3′-OH is possibly the first step for C-3′ dehydroxylation. The gentamicin biosynthetic gene *gntI*, encoding an aminoglycoside phosphotransferase, was cloned from *Micromonospora echinospora* ATCC15835 and overexpressed in *Escherichia coli*. The resulting phosphotransferase was purified, and the kinetic parameters for Kanamycin A, Kanamycin B, Neomycin B and Amikacin were determined. Elucidation of NMR data of phosphorylated kanamycin B has unambiguously demonstrated a regiospecific phosphorylation of 3′-hydroxyl of the 6-aminohexose ring. The results described here partly confirm that the 3′-dehydroxylation step is preceded by a 3′ phosphorylation step. It is predicted that GntI belongs to a new aminoglycoside phosphotransferase group involved with aminoglycoside antibiotics biosynthesis pathway.

Gentamicins is a group of broad-spectrum aminoglycoside antibiotics isolated from *Micromonospora*, including gentamicin C_1, C_{1a} and C_2.[1,2] Gentamicins act by binding irreversibly to the bacterial 30S ribosomal subunit protein and 16S rRNA, and preventing the formation of the initiation complex with messenger RNA.[3] For 50 years Gentamicins were used extensively for the treatment of many bacterial infections, and the additional anti-viral properties of some gentamicin-conjugants have also been demonstrated.[4,5] The gentamicins are composed of 2-deoxystreptamine (2-DOS) substituted at C-4 with a purpurosamine sugar and at C-6 with a garosamine sugar. Kanamycin, another important clinical glyamino-type antibiotic isolated from *Streptomyces kanamyceticus*, is often modified by the phosphorylase from the clinic resistant bacteria, while Gentamicin which lacks 3′-hydroxyl group can avoid the attack from the modification enzymes.[6] Gentamicins and kanamycin share a similar biosynthetic pathway. However, the lack of the key tailoring enzymes in kanamycin biosynthetic gene cluster leads to the structural difference between kanamycin and gentamicins, including the retaining and leaving of C-3′ hydroxyl group. Consequently, C-3′ dehydroxylation is a key step in gentamicins biosynthesis (Fig. 1).[7–9] Identification of the dedicated biosynthetic enzyme thus is important for understanding the biosynthetic pathway of gentamicins.

Gentamicins belong to a group of natural products called 2-deoxystreptamine-containing aminoglycoside antibiotics. The gentamicins biosynthetic gene cluster from *Micromonospora* has been reported.[10] The early biosynthetic steps involved in the formation of core structure of gentamicins have been determined. At first, *N*-acetyl-D-glucosamine is added to 2-DOS by GntD/GntZ to form a paromamine structure. The gentamicin C pseudotrisaccharide backbone is thought to derive from the addition of D-xylohexose to paromamine by a glycosyltransferase (GntD) and subjected to a series of modifications by tailoring enzymes to form a key intermediate JI-20A. Additional modification enzymes led to the biosynthesis of the final gentamicins.[11,12]

GntI is a unique putative phosphorylase found in the gentamicins biosynthetic gene cluster.[9] The phosphorylase involved in aminoglycoside antibiotics is primarily a class of modifying enzymes which *O*-phosphorylate the antibiotics. Interestingly, unlike most other aminoglycoside-modifying enzymes which have been cloned from clinical isolates, three aminoglycoside phosphotransferase (APH)-encoding genes were found in the gene clusters responsible for the biosynthesis of gentamicin, sisomicin and fortimicin, respectively. The similar organizations of genes in the clusters for gentamicin, sisomicin and fortimicin biosynthesis demonstrate their similar structures and biosynthetic routes.[13] In the complementary experiment, *forP* is able to restore production of fortimicin

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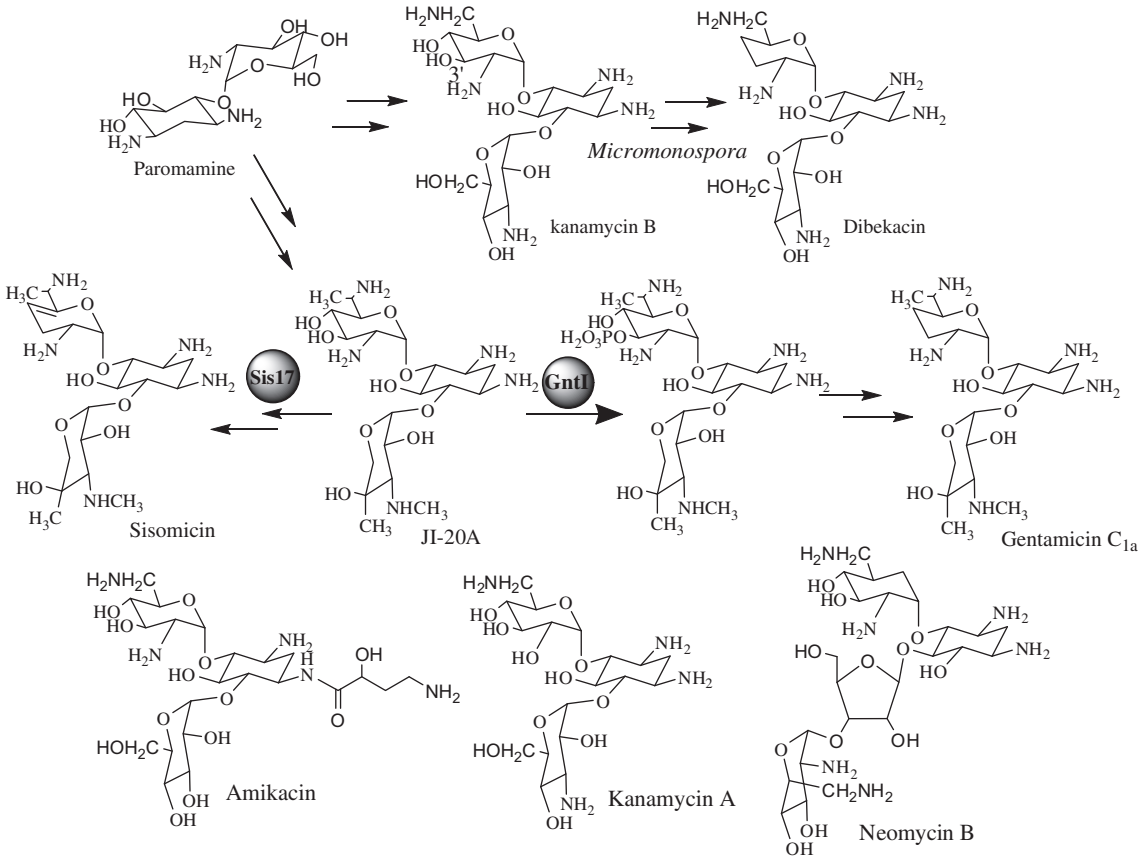

Figure 1. The biosynthesis pathway of gentamicin, sisomicin and kanamycin.

A of the mutants which is deficient in the bisdehydroxylation of FTM-KK1 (Supplementary Fig. 1). [14] Consequently, ForP is necessary for the 3′,4′-didehydroxylation of FTM-KK1. Protein sequence alignment of GntI, Sis17 and ForP shows quite high sequence similarity with a number of aminoglycoside-*O*-phosphoryltransferases of the APH (3′) family-enzymes (Fig. 2A). However, the similar phosphorylase gene has never been found in biosynthesis gene cluster of other aminoglycoside antibiotics containing 3′-OH group.[15] This leads to a hypothesis that a phosphorylation of 3′-OH could be involved in the bisdehydroxylation C-3′ and C-4′. Moreover, the lack of the key intermediate JI-20A has hampered the studies of in vitro activity of the key enzymes involved with these biosynthetic steps. In the study reported here, we reconstituted GntI in *Escherichia coli* BL21 (DE3) and used JI-20A analogue, kanamycin B, to determine the biochemical characterization of GntI as the C-3′ phosphorylase. In vitro assay, the recombinant enzyme can efficiently catalyze the phosphorylation of a series of aminoglycoside antibiotics, including kanamycin A, kanamycin B, neomycin B and amikacin.

In order to investigate the biochemical properties of GntI, the gene was subcloned in frame with a six-histidine-tag sequence into expression vector and expressed the His-tagged (N-terminal) fusion protein in *E. coli* BL21. At first, the *gntI* gene (GenBank accession number AY524043) was amplified from genomic DNA of *Micromonospora echinospora* ATCC15835 by PCR (*Supplementary* Fig. 2). Then, the PCR product was purified, digested with NdeI and HindIII and then cloned into pET-28a(+) (Novagen), which was cut with the same restriction endonucleases to yield pET-28a-*gntII*. The gene was then sequenced in order to ensure that no mutations occurred during the PCR procedure.

The plasmids were transformed into *Escherichia coli* BL21(DE3) and expressed at 28 °C. SDS–PAGE analysis of the soluble and insoluble fractions of the cell lysate showed that the bacteria containing recombinant plasmid pET-28a-gntI produced a substantial amount of expected recombinant protein, but this protein was not detectable in the culture of the bacteria containing the parent vector pET-28a. The recombinant GntI protein was initially purified with Ni-NTA resin chromatography. Degraded and/or nonspecifically bound polypeptides in elutes from Ni-NTA resin column were removed by subsequent gel filtration chromatography (GFC). Finally we obtained 12 mg of purified protein from a 2-liter culture. The purified GntI protein shows a Mr of about 30,000, in agreement with its calculated Mr (Fig. 3).

Kinetic analysis of GntI was performed by the pyruvate kinase/lactate dehydrogenase coupled assay.[16–18] The assay measures the rate of NADH absorbance decrease at 340 nm, which is proportional to the rate of steady-state ATP consumption. The oxidation of NADH was followed by continuously monitoring the absorbance at 340 nm. A typical assay mixture contained 50 mM Tris–HCl buffer (pH 7.5), 40 mM KCl, 10 mM $MgCl_2$, 0.16 mM NADH, 2.5 mM PEP, 1 mM ATP, 20 μ/mL pyruvatekinase, 20 μ/mL lactate dehydrogenase and varied concentrations of the aminoglycoside analog. The mixture was preincubated at 37 °C for 5 min and all reactions were started by the addition of the enzyme (10 nM final). Aminoglycosides consisting of three rings (kanamycin A, kanamycin B and amikacin) and four rings (neomycin B) were selected for these experiments. K_m and k_{cat} values were determined by nonlinear regression analysis using Prism 5.0. The K_m values for the aminoglycosides, with the exception of amikacin, were typically in the lower micromolar range (1–20 μM). Furthermore, the values of k_{cat} are generally within twofold of each other. These observations are