Enzymes

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The Last Step of Kanamycin Biosynthesis: Unique Deamination Reaction Catalyzed by the α-Ketoglutarate-Dependent Nonheme Iron Dioxygenase KanJ and the NADPH-Dependent Reductase KanK**

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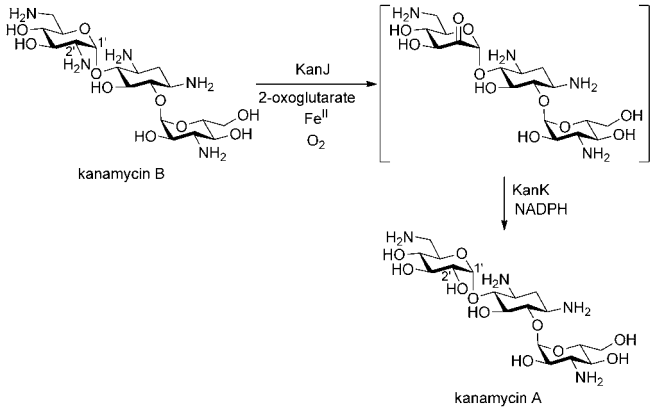
Kanamycin (Scheme 1) is a clinically important aminoglycoside antibiotic, containing 2-deoxystreptamine (2DOS), and is produced by *Streptomyces kanamyceticus*.[1] However, because bacteria are known to become resistant to this antibiotic, investigations into the bacterial resistant mechanism led to the development of semisynthetic kanamycin derivatives such as dibekacin, amikacin, and arbekacin (see Figure S1 in the Supporting Information).[2] These derivatives show good activities against kanamycin-resistant bacteria and are used as chemotherapeutic agents against resistant bacteria of clinical importance.



Scheme 1. KanJ and KanK reaction with kanamycin B to afford kanamycin A.

The biosynthetic gene cluster for kanamycin (*kan*) was identified from *Streptomyces kanamyceticus* in 2004 by Japanese,[3] Korean,[4] and German groups.[5] Bioinformatics analysis of the kanamycin gene cluster with other 2DOS-containing aminoglycoside biosynthetic genes revealed that the *kan* gene cluster encodes two unique enzymes KanJ, a putative phytanoyl-CoA dioxygenase and KanK, a putative NADP(H)-dependent oxidoreductase, both of which were presumed to be involved in kanamycin A biosynthesis.[6] Among 2DOS aminoglycosides, only kanamycin A has a unique hydroxy group at the C2′-position whereas the others possess an amino group at the same position (see Figure S1 in the Supporting Information). This particular attribute of kanamycin A has led us to propose two exclusive proteins, KanJ and KanK, as being responsible for the transformation from kanamycin B to kanamycin A, presumably with an oxidation/reduction at the C2′-position. To confirm the functions of KanJ and KanK, the enzymatic activity of recombinant KanJ and KanK proteins were investigated.

The *kanJ* and *kanK* genes were amplified by PCR and cloned into the pColdI expression vector to overexpress in *E. coli*. To obtain soluble protein, KanK was coexpressed with molecular chaperones GroES and GroEL. The recombinant KanJ and KanK proteins were purified with nickel-affinity chromatography and were characterized for their enzymatic reaction with kanamycin B as a substrate.

The enzyme reaction products were treated with 2,4-dinitrofluorobenzene (DNFB) to convert aminoglycosides into *N*-dinitrophenyl (DNP) derivatives, which show absorption at $\lambda = 350$ nm (Figure 1). As a result, it was found that

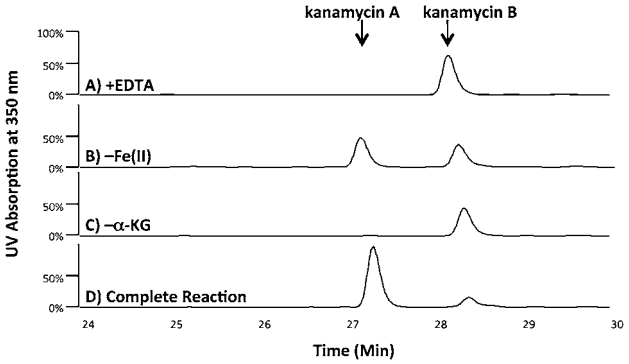


Figure 1. HPLC analysis of the KanJ/KanK reaction products from kanamycin B. The elution was monitored at $\lambda = 350$ nm. The scale of traces is normalized. A) Complete assay with EDTA. B) Complete assay without Fe^{II}. C) Complete assay without α-KG. D) Complete assay. Assay conditions: KanJ (20.3 μM) and KanK (8.6 μM) were incubated with kanamycin B (0.5 mM), α-KG (1 mM), $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$ (1 mM), and NADPH (1 mM) at 28 °C for 15 h. EDTA (1 mM) instead of Fe^{II} was added for reaction data shown in A. See experimental section for HPLC conditions.

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kanamycin B was efficiently converted into kanamycin A in the presence of 2-oxoglutarate (α-ketoglutarate; α-KG), $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$, and NADPH. Evidently, maximal KanJ activity was shown to be dependent on exogenous ferrous ions, although residual activity was also observed in the absence of exogenous Fe^{II}. This dependency was likely the result of small amounts of enzyme-bound ferrous ions that were retained during enzyme purification. However, the addition of EDTA completely abolished the activity. Also, in the absence of α-KG, formation of kanamycin A was not observed, thus suggesting that KanJ requires these cofactors for the enzymatic activity as a standard Fe^{II}/α-KG-dependent dioxygenase.[7] The cofactor requirement was expected from the homology analysis of KanJ appearing in this family of dioxygenase (see Figure S2 in the Supporting Information). The KanJ and KanK reaction product from kanamycin B was additionally isolated from a large-scale enzyme reaction (15 mL, 15.5 mg) and the structure of the isolated compound (7 mg) was confirmed as kanamycin A by NMR spectroscopy and FAB/MS (see Figures S4–S7).

When NADH was added to the reaction instead of NADPH, kanamycin A was not detected, thereby indicating that KanK prefers to use NADPH (see Figure S8 in the Supporting Information). Even in the absence of NADPH or KanK, kanamycin B was efficiently consumed by KanJ and a new DNP derivative having a *m*/*z* 820 was detected by LC/ESI/MS (see Figures S8 and S9), thus indicating that the presumable KanJ reaction product, 2′-oxokanamycin, was decomposed into a pseudo-disaccharide during derivatization with DNFB under basic conditions. Because any attempt to isolate the KanJ reaction product has failed so far, the KanJ enzymatic reaction was treated with $NaBH_4$ or $NaBD_4$ to clarify the expected ketone formation. ^{1}H NMR spectroscopy and LC/ESI/MS analysis revealed that this coupled reaction indeed afforded kanamycin A in addition to its diastereomer (Figure 2 and Figure S10). Furthermore, incorporation of a deuterium atom into the C2′-position was detected by ^{1}H NMR spectroscopy as confirmed by the lack of a coupled signal with H1′ (Figure 2) and also by ^{2}H NMR spectroscopy (Figure S11). This result clearly demonstrated that the KanJ-generated 2′-oxokanamycin was reduced by $NaBD_4$. Another product, ammonia, was also clearly detected by the use of a coupling assay with glutamate dehydrogenase (Figure S12).

These results support the synthesis of 2′-oxokanamycin and release of ammonia by KanJ. Consequently, in the KanJ/KanK coupling reaction, KanK appeared to stereoselectively reduce 2′-oxokanamycin in the presence of NADPH to afford kanamycin A (Scheme 1). The possibility of the reverse reaction from kanamycin A to 2′-oxokanamycin by KanK with $NADP^+$ was not observed (data not shown). Therefore, the equilibrium of the KanJ/KanK reaction appeared to be largely on the side of kanamycin A production, presumably because of the $NADP^+$ formation and the release of ammonia. Notably, this mechanism is distinct from that of the well-known adenosine deaminase which catalyzes hydration of the adenine ring with subsequent release of ammonia, thus forming a carbonyl group where no redox reaction is involved.[8] In the transformation of kanamycin B into kanamycin A, oxidation and reduction is required to remove an amino group and to form a hydroxy group, respectively. The first step of this transformation, catalyzed by KanJ, should be rather comparable to the oxidative deamination by glutamate dehydrogenase which utilizes NAD^+/$NADP^+$ as an oxidant, and the second reaction, involving KanK, is similar to the reverse reaction with a "keto"-compound as the substrate.[9]

Two possible reaction mechanisms were hypothesized to explain the process of the ketone formation by KanJ: A) the reactive $[Fe^{IV}{=}O]$ species breaks the inactive C2′–H2′ bond in kanamycin B homolytically to produce the kanamycin B

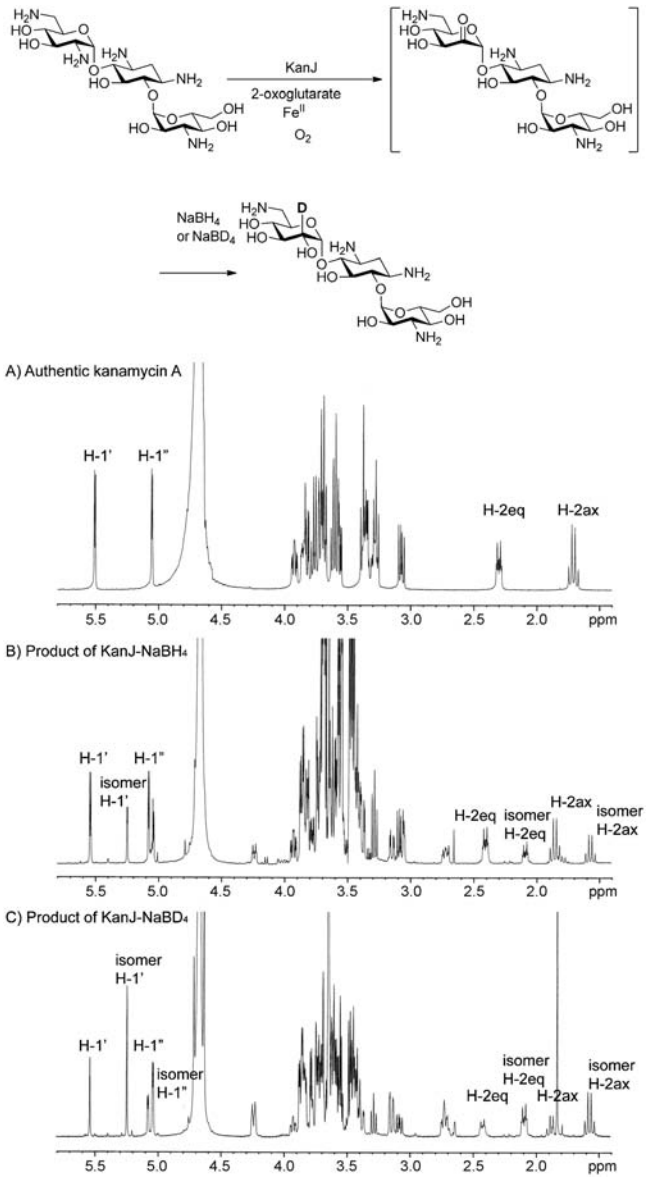
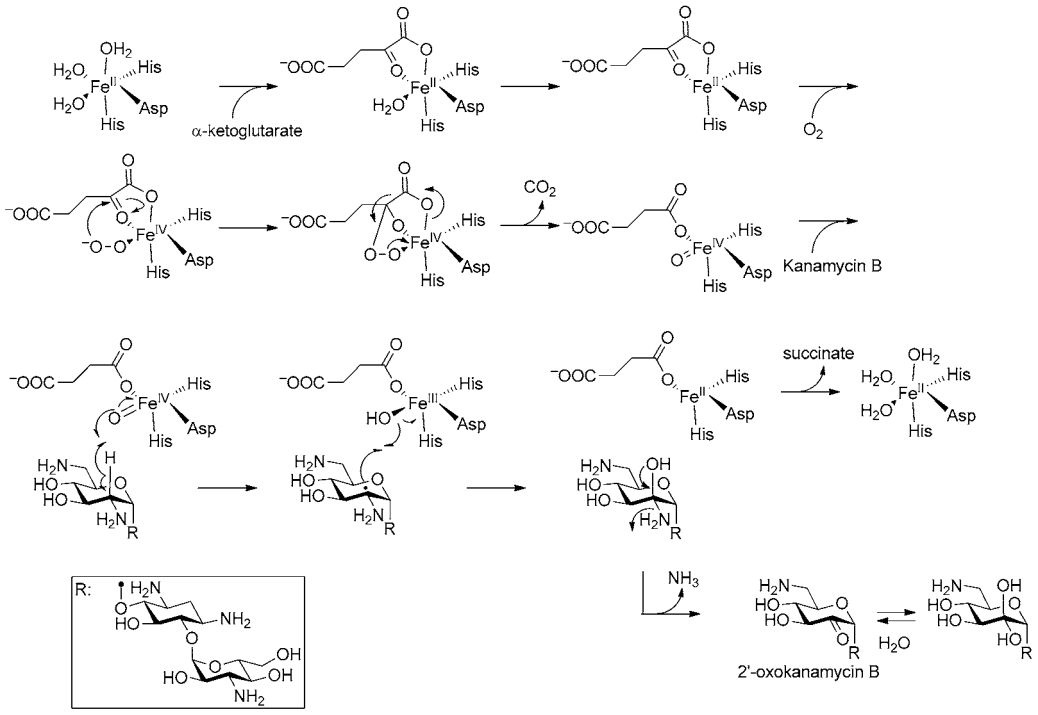


Figure 2. ^{1}H NMR spectra of the KanJ reaction product reduced with $NaBH_4$ or $NaBD_4$. A) Authentic kanamycin A. B) Isolated product of KanJ-$NaBH_4$. C) Isolated product of the reaction with KanJ and $NaBD_4$ (500 MHz, D_2O). Assay conditions: KanJ (25.8 μM) was incubated with kanamycin B (2.1 mM), α-KG (6 mM), $(NH_4)_2Fe(SO_4)_2{\cdot}6H_2O$ (6.8 mM) at 28 °C for 48 h. $NaBD_4$ or $NaBH_4$ was added excessively into the enzyme assay solutions and stirred overnight at 4 °C. See experimental section for isolation procedure.



Scheme 2. Proposed KanJ reaction mechanism.

radical at C2′ and facilitates its own reduction to an Fe^{III}-OH species. Fe^{III}-OH provides a hydroxyl radical to afford a hemiaminal intermediate, which is simultaneously converted into 2′-oxokanamycin (Scheme 2); B) the reaction is initiated by the radical attack of a ferryl oxo species onto the lone pair of the nitrogen atom at C2′ to form a nitrogen cation radical intermediate, which turns into an imine intermediate that is easily hydrolyzed to a ketone (see Scheme S1 in the Supporting Information). The ketone intermediate formed through either pathway is then subsequently reduced to form kanamycin A by KanK. To distinguish these reaction mechanisms the KanJ reaction coupled with KanK was conducted under an ^{18}O-enriched atmosphere. From the LC/ESI/MS of the enzymatic reaction products, 7% of the produced kanamycin A was found to be labeled with ^{18}O (Table 1 and Figure S13).

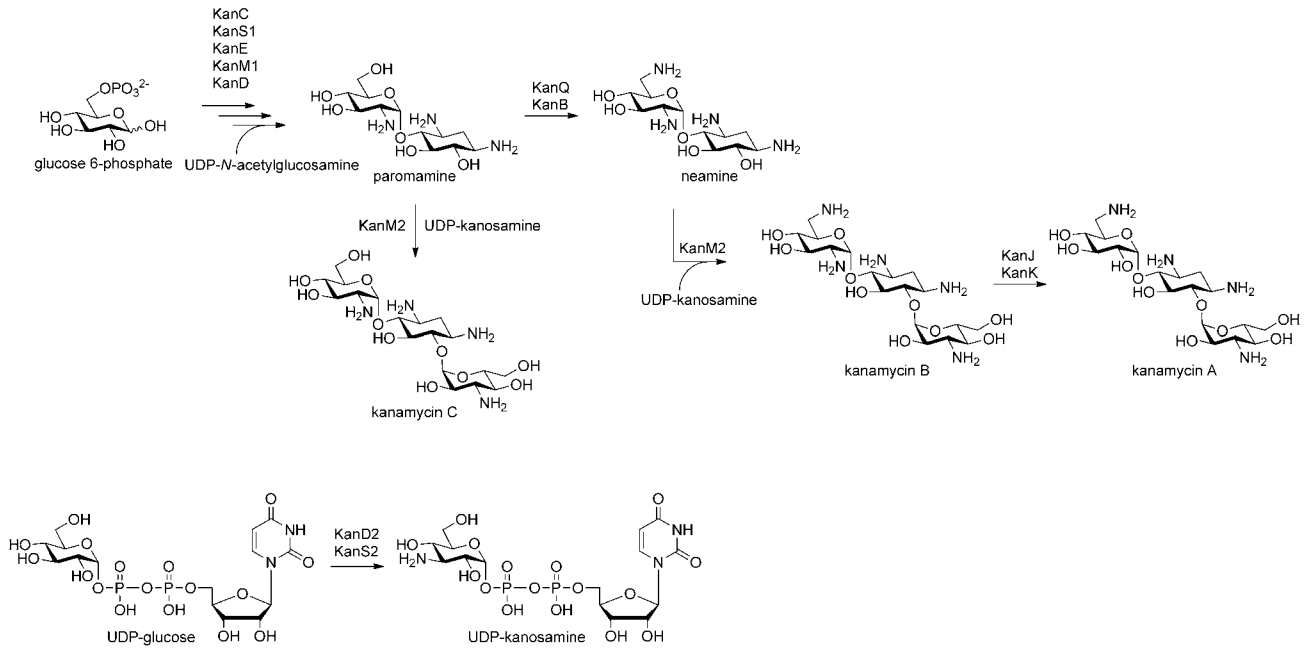
This result clearly suggested that KanJ catalyzes the direct hydroxylation to afford a hemiaminal intermediate just as in the case of the usual α-KG-dependent hydroxylase.[7a] The incorporation of oxygen atoms from O_2 into kanamycin is indicative that KanJ is a α-KG-dependent dioxygenase, which usually transfers one oxygen atom to the substrate and another to α-KG, thus resulting in formation of succinate. The small amount of ^{18}O incorporation from molecular $^{18}O_2$ appeared to be caused by the equilibrium exchange involving the ^{16}O from the water and the ketone intermediate 2′-oxokanamycin B (Scheme 2). Thus, this equilibrium event was confirmed by analyzing the enzymatic reaction in the presence of 50% ^{18}O-labeled water. As expected, a significant amount of ^{18}O (19%) was clearly incorporated into kanamycin A (Table 1 and see Figure S13 in the Supporting Information), thus indicating that an equilibrium event between the ketone and acetal intermediate with water occurred. The hemiaminal formation catalyzed by KanJ is a very unique strategy to remove an amino group (Scheme 2). This reaction mechanism is different from that of the pyridoxal 5′-phosphate (PLP)-dependent transamination, in which the formed aldimine intermediate between the amino group and PLP is hydrolyzed into a ketone and pyridoxamine 5′-phosphate (PMP), and the amino group in PMP is then transferred to another ketone-containing molecule such as 2-oxoglutarate to complete the catalytic cycle. The KanJ/KanK reaction does not represent a reverse reaction scheme for installation of an amino group by a combination of dehydrogenase and aminotransferase activities as is known in aminoglycoside biosynthesis.[10]

Table 1: Incorporation of ^{18}O into kanamycin A from molecular $^{18}O_2$ and $H_2{}^{18}O$ during the KanJ reaction.

Conditions	*m/z* 1147	*m/z* 1148	*m/z* 1149	*m/z* 1150	Incorporation[%]
Calc.	100	47	16.4	4.9	–
$^{16}O_2/H_2{}^{16}O$	100[a]	51.6[a]	20[a]	5.9[a]	0
$H_2{}^{18}O$	100[a]	62.7[a]	**39.2**[a]	17.6[a]	**19.2**
$^{18}O_2$	100[a]	49.5[a]	**27.5**[a]	13.2[a]	**7.5**

[a] Relative intensities of the peaks in the ESI/MS (negative mode) spectra of the kanamycin A DNP derivative. Each data point was taken in triplicate.

To date, it has been reported that five conserved biosynthetic enzymes among 2DOS-containing biosynthetic gene clusters (KanC, 2-deoxy-*scyllo*-inosose (2DOI) synthase, KanS1, Gln:2DOI aminotransferase, KanE, NAD dependent 2-deoxy-*scyllo*-inosamine dehydrogenase, KanM1, UDP-GlcNAc:2DOS glycosyltransferase, and KanN, 2′-*N*-acetylparomamine deacetylase) encoded in the *kan* gene cluster are responsible for the paromamine formation by heterologous expression of the genes in *Streptomyces lividans*.[11] The function of KanM2 has been clarified as a glycosyltransferase that catalyzes the glycosylation of paromamine with UDP-glucose (UDP-Glc) to yield 3″-deamino-3″-hydroxykanamycin C in our previous report.[12] A recent report on the kanamycin biosynthetic pathway indicated that KanM2 (stated as KanE, see a review[13] to compare the corresponding gene) recognizes UDP-kanos-



Scheme 3. Biosynthetic pathway of kanamycins.

amine to afford kanamycin C with better activity than UDP-Glc as a glycosyl donor.[14] In addition, two sets of dehydrogenase and aminotransferase pairs: KanD2/KanS2 and KanQ/KanB (stated as KanC/KanD and KanI/KanL) are suggested to be involved in the installation of amino groups at C3″ and C6′, respectively, of kanamycin A. Therefore, our finding here that KanJ/KanK catalyzes the transformation of kanamycin B into kanamycin A completes the last step of kanamycin A biosynthesis (Scheme 3). This linear biosynthetic pathway for kanamycin A appears to be dominant in *S. kanamyceticus* since kanamycin A was isolated as a major dead-end natural product. The substrate promiscuities and the different conversion rates of the Kan biosynthetic enzymes and efflux proteins would account for the production of kanamycin B and kanamycin C from the pathway. Bioinformatics analysis indicates that the conserved biosynthetic enzymes among 2DOS-containing aminoglycoside biosynthetic gene clusters function as the same catalysts, and that pathway specific enzymes like KanJ and KanK appear to add structural uniqueness into the common skeleton of aminoglycosides. In fact, many unassigned hypothetical proteins are encoded in the identified aminoglycoside biosynthetic gene clusters and remain to be characterized as unique modification enzymes in the biosynthesis of aminoglycoside antibiotics.[6]

In summary, this report describes for the first time KanJ as a unique Fe^{II}/α-KG dependent dioxygenase protein and confirms KanK as a NADPH-dependent ketoreductase. The evidence clearly supports the idea that KanJ and KanK are responsible for the formation of kanamycin A in the final synthetic step of kanamycin. An in-depth characterization of KanJ including substrate specificity is currently in progress.

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