

Purification and Characterization of 2-Deoxy-*scyllo*-inosose Synthase Derived from *Bacillus circulans*. A Crucial Carbocyclization Enzyme in the Biosynthesis of 2-Deoxystreptamine-containing Aminoglycoside Antibiotics

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The biosynthesis of 2-deoxystreptamine, the central aglycon of a major group of clinically important aminoglycoside antibiotics, commences with the initial carbocycle formation step from D-glucose-6-phosphate to 2-deoxy-*scyllo*-inosose. This crucial step is known to be catalyzed by 2-deoxy-*scyllo*-inosose synthase, which has not yet been characterized so far. Reported in this paper is the first purification of 2-deoxy-*scyllo*-inosose synthase from butirosin-producing *Bacillus circulans* SANK 72073 to electrophoretic homogeneity. The enzyme was isolated as a heterodimeric protein comprising from a 23 kDa- and a 42 kDa polypeptide chains. The K_m of the enzyme for D-glucose-6-phosphate was estimated to be 9.0×10^{-4} M and that for NAD^+ 1.7×10^{-4} M, k_{cat} for D-glucose-6-phosphate being 7.3×10^{-2} s $^{-1}$. The presence of Co^{2+} was essential for the enzyme activity, but Zn^{2+} was totally inhibitory. While the reaction mechanisms are quite similar, 2-deoxy-*scyllo*-inosose synthase appears to be distinct from dehydroquinase synthase in the shikimate pathway, with respect to the quaternary structure, metal ion requirement, and the kinetic parameters.

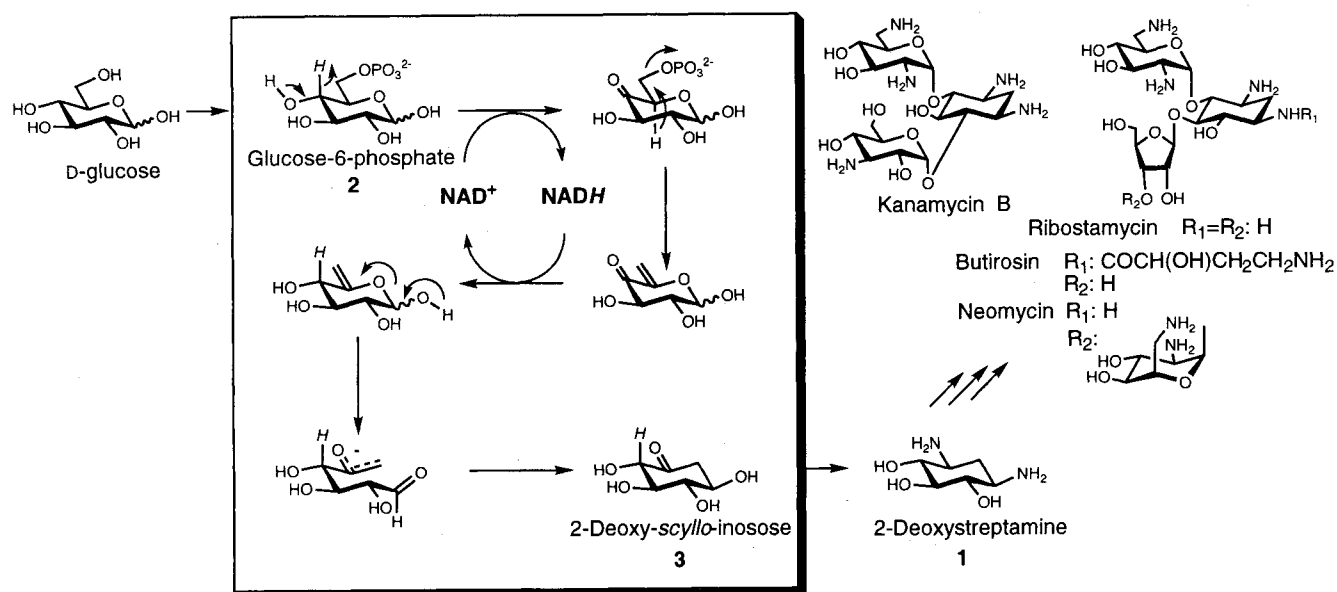
Aminoglycoside antibiotics are among those clinically important and a major structural feature is the existence of characteristic aglycons^{2,3}. In view of the structure and biosynthesis of aminocyclitol aglycons, these antibiotics are classified into two classes. One is those that contain a fully-substituted aminocyclitol. The aminocyclitols in this class were shown or proposed to be biosynthesized from *myo*-inositol^{4~6}, which is ubiquitous in animals, plants, and microorganisms. Significant progress had appeared for the biosynthetic studies of inositol-derived aminoglycoside antibiotics such as streptomycin⁷ and fortimicin⁸) by genetic analysis. On the other hand, the other major class of aminoglycoside antibiotics including kanamycin, neomycin, ribostamycin, butirosin, *etc.* contain a unique 2-deoxystreptamine (DOS, **1**) aminocyclitol, which is found only in these antibiotics and is thus a typical product of microbial secondary metabolism. The biosynthesis of DOS-containing aminoglycoside antibiotics has been extensively studied, mostly by the

use of whole cells of the producing microorganisms as well as non-producing blocked (idiotrophic) mutants^{9~12}). However, the enzymes involved in the biosynthesis of these antibiotics have mostly been remained unclear except for some particular enzymes, *e.g.* transamination enzymes of relevant inososes^{13,14}).

Among the whole biosynthesis processes of the DOS-containing antibiotics which has been attracting attention as mentioned above, 2-deoxy-*scyllo*-inosose synthase (DOI synthase) reaction is the crucial starter process, in which the intramolecular carbocyclization of D-glucose-6-phosphate (G-6-P, **2**) is catalyzed to give the first non-aminogenous cyclitol 2-deoxy-*scyllo*-inosose (DOI, **3**)^{9,10,15}). DOI is ultimately transformed into DOS by several manipulations including transamination, *etc.*^{2,3,13,14}). We have recently elucidated the mechanism of this key enzyme reaction, as shown in Fig. 1, by the use of partially purified enzymes derived from butirosin-producing *Bacillus circulans* SANK 72073 and neomycin-

* Biochemical studies on 2-deoxy-*scyllo*-inosose, an early intermediate in the biosynthesis of 2-deoxystreptamine Part VIII. For Part VII, see ref. 1.

Fig. 1. The overall biosynthetic pathway of 2-deoxystreptamine-containing aminoglycoside antibiotics and the mechanism of 2-deoxy-*scyllo*-inosose synthase reaction [blackboxed].



producing *Streptomyces fradiae* IFO 13147^{1,16,17}). The multi-step process includes the first oxidation at the C-4 position of G-6-P, and an elimination of a phosphate group from the activated ulose is followed to form an enol or enolate intermediate. Subsequent reduction at C-4 and the last aldol-type intramolecular condensation between C-1 and C-6 give rise to DOI.

In view of the six-membered carbocyclic ring construction in the living systems, this DOI synthase reaction (a secondary metabolism) appears to be mechanistically similar to the dehydroquininate synthase (DHQ synthase) reaction in the shikimate pathway (a primary metabolism)^{18~23}. Comparative studies of DOI synthase and DHQ synthase from chemical and biochemical viewpoints are quite intriguing and important to get further insight into each enzyme reaction mechanism, and may give rise to some clue to the understanding of molecular evolution of the microbial secondary metabolism.

In this paper, we report for the first time the purification and characterization of DOI synthase from the cells of butirosin-producing *Bacillus circulans* SANK 72073.

Materials and Methods

Bacterial Strain and Culture Conditions

A butirosin-producing organism *Bacillus circulans* SANK 72073 was maintained as described previous-

ly¹⁶). A saline suspension (0.9% NaCl, 1 ml) of the *B. circulans* cells from a stock slant was inoculated to 200 ml of nutrient-glycerol medium (1% beef extract, 1% peptone, 0.5% NaCl, 1% glycerol) in a 500-ml Erlenmeyer flask. Precultures were performed on a rotary shaker (180 rpm) at 28°C until OD₆₆₀ reached to 7.0~9.0. A portion (1 ml) of the preculture was used for inoculation of the growth culture (200 ml × 24 flasks). Cultures were performed in the same way until OD₆₆₀ reached to 8.0~9.0. The cells were harvested by centrifugation (6,000 × *g* for 30 minutes, 4°C), and were washed with 50 mM Tris-HCl buffer solution (pH 7.7) (6,000 × *g* for 30 minutes, 4°C). The cells were stocked at -70°C until use.

Reagents

D-glucose-6-phosphate di-potassium salt (G-6-P) was purchased from Sigma. NAD⁺ was purchased from Oriental Yeast Co. (Tokyo). The 2-deoxy-*scyllo*-inosose synthetic standard was prepared according to the previously reported method²⁴). All other chemicals were of the highest grade commercially available.

Physical and Chemical Measurement

The protein content of an enzyme preparation was estimated by the Lowry's method²⁵) with bovine serum albumin as a standard.

Instrumentation

High pressure liquid chromatography (HPLC) was performed on a Hitachi L-6000 pump equipped with a Hitachi L-4000 UV-detector and a Hitachi D-2500 integrator for the assay of enzyme activity. For the enzyme purification, FPLC (Pharmacia Biotech) was used.

2-Deoxy-scyllo-inosose Synthase Assay

The DOI synthase activity was detected by modifying the previously described HPLC method²⁶. An aliquot (70 μ l) of an enzyme preparation was mixed with 10 μ l of 50 mM G-6-P, 10 μ l of 50 mM NAD⁺ and 10 μ l of 50 mM CoCl₂ in 50 mM Tris-HCl buffer (pH 7.7, final volume was 100 μ l, final concentration of G-6-P, NAD⁺, and Co²⁺ being 5 mM). Incubation was carried out at 46°C for 1 hour. The reaction was terminated by adding 100 μ l of CH₃OH. To the resulting mixture, 20 μ l of *O*-(4-nitrobenzyl)hydroxylamine hydrochloride (NBHA) in pyridine (5 mg/ml) was added in order to form a spectrophotometrically visible oxime derivative. The whole mixture was then heated to 60°C for 1 hour. Then, the solvent was removed by flushing the air. The residue was dissolved in 2 ml of a mixture of CHCl₃ and CH₃OH (20:1) and the mixture was applied to Sep-Pak Plus Silica. The unreacted residual NBHA was first eluted with 5 ml of a mixture of CHCl₃ and CH₃OH (15:1). The *O*-(4-nitrobenzyl)oxime derivative of DOI was then eluted with 5 ml of a mixture of CHCl₃ and CH₃OH (5:1). After the eluate was evaporated, the residue was dissolved in 100 μ l of CH₃OH. A portion (5 μ l) of the solution was injected into the aforementioned HPLC apparatus equipped with a TSK-gel ODS-80TM CTR column (4.6 mm i.d. \times 10 cm, TOSOH). The elution was monitored by UV absorbance at 262 nm. The eluent was consisted of 20% of CH₃OH in deionized water. The amount of DOI *O*-(4-nitrobenzyl)oxime was quantitated by the standard curve method. One unit of the enzyme activity was defined as the production of 1 nmol of DOI per minute.

Purification of 2-Deoxy-scyllo-inosose Synthase

The harvested *B. circulans* cells (80 g of wet cells) were suspended in 240 ml of a 50 mM Tris-HCl buffer (pH 7.7) containing 50 μ M phenylmethanesulfonyl fluoride (PMSF) and 50 μ M EDTA and then the whole was sonicated by a Branson sonifier Type 250 repeatedly (10 minutes \times 10 times) at 0°C in an iced water bath. The sonicate was centrifuged at 6,000 \times *g* for 30 minutes and the resulting supernatant was then fractionated by

(NH₄)₂SO₄ precipitation in a standard manner.

The precipitate obtained by 40~70% (NH₄)₂SO₄ saturation was dialyzed at 4°C overnight against a 50 mM Tris-HCl buffer containing 50 μ M PMSF and 50 μ M EDTA (pH 7.7), and the dialysate was loaded onto a column (2.5 \times 15 cm) of DEAE-cellulofine A-800 (Chisso) equilibrated with 50 mM Tris-HCl (pH 7.7). The adsorbed proteins were eluted by the same buffer with linear gradient of NaCl concentration from 0 M (300 ml) to 0.2 M (300 ml). The DOI synthase activity was observed in the fractions of approximately 0.1 M NaCl. The active fractions were concentrated into *ca.* 10 ml by Centriprep-10 (Amicon) (centrifugation 2,000 \times *g* for 30 minutes several times). The concentrate was then loaded onto a column (2.5 \times 8.5 cm) of TSK-gel AF-Blue Toyopearl 650 ML (TOSOH) equilibrated with 50 mM Tris-HCl (pH 7.7). The adsorbed proteins were eluted with the same buffer containing 1 M NaCl. The active fractions were concentrated into *ca.* 2 ml in the same manner. The concentrate was loaded further onto a Hi Load 26/60 Superdex 200 pg (prep grade, FPLC) equilibrated with 50 mM Tris-HCl (pH 7.7) containing 0.1 M NaCl. The active fractions were again concentrated into *ca.* 2 ml in the same manner. The concentrate was subsequently loaded onto a Mono Q HR 10/10 (FPLC) equilibrated with 50 mM Tris-HCl (pH 7.7) containing 0.1 M NaCl. The adsorbed proteins were eluted by the same buffer with linear gradient of salt concentration from 0.13 to 0.16 M NaCl. The enzyme activity was observed in the fractions of *ca.* 0.14 M NaCl. All the operation were carried out at 4°C, except for FPLC separation. Having been frozen, the enzyme was fully active after six months' storage at -75°C.

Molecular-weight Determination

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the literature procedure²⁷, and the proteins were visualized by using Coomassie brilliant blue staining. The molecular-weight standards used (from Bio-Rad) were lysozyme (14,400), trypsin inhibitor (21,500), carbonic anhydrase (31,000), ovalbumin (45,000), serum albumin (66,200), and phosphorylase b (97,400). Native polyacrylamide gel electrophoresis (Native-PAGE) was performed also according to the literature procedure²⁸.

Molecular-weight determination under non-denaturing conditions was carried out by Superdex 200 gel filtration chromatography. The column was calibrated with standard (from BDH Chemicals Ltd.) containing carbonic anhydrase (30,000), ovalbumin (45,000),

ovotransferrin (77,000).

Chromatographic Analysis

Native DOI synthase was loaded onto TSK-gel G3000SW Glass column (TOSOH) equilibrated with 50 mM Tris-HCl buffer (pH 7.7) containing 0.1 M NaCl. SDS-treated DOI synthase was loaded onto the same column equilibrated with the 50 mM Tris-HCl buffer (pH 7.7) containing 0.1% SDS.

DOI synthase was applied to an isoelectric focusing column (Mono P HR 5/20) equilibrated with 25 mM Tris-HCl buffer (pH 7.0). The adsorbed DOI synthase was eluted by polybuffer 74 (Pharmacia Biotech)-HCl (pH 3.7).

Characterization of the DOI Synthase

DOI synthase was characterized under the aforementioned enzyme assay conditions by varying pH, metal concentration, and temperature. Optimum pH was determined by the assay in a 50 mM Tris-maleate-NaOH buffer between 5.5 to 8.5. To study the requirement of divalent metal ion, each metal chloride including $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$, CaCl_2 , ZnCl_2 , and $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ was added in a final concentration of 5 mM. Metal-free condition was made by adding EDTA (final concentration 1 mM). For the determination of the optimum reaction temperature, the enzyme reaction was carried out at 25, 30, 37, 42, 46 and 50°C.

Steady-state Kinetic Analysis

DOI synthase reaction was analyzed by varying the concentration of G-6-P from 125 to 2000 μM or the concentration of NAD^+ from 62.5 to 1000 μM .

Results and Discussion

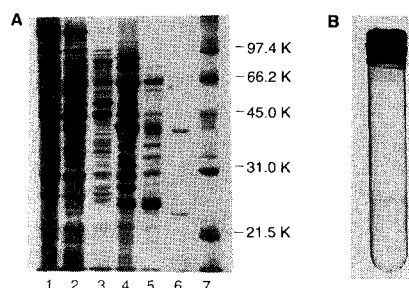
Purification of the Enzyme

It seems worth to point out at first that we encountered some difficulty in obtaining DOI synthase activity with good reproducibility from the *B. circulans* SANK 72073 cells. The harvesting time of *B. circulans* cells was very critical. The best reliable results were obtained by harvesting the cells just before the idiophase started. Either in the tropophase or in the latter idiophase, total enzyme activity was very low (data not shown). This observation of the DOI synthase production is well accord with a general trend of the expression of the microbial secondary metabolism.

The DOI synthase from *B. circulans* SANK 72073 was

Fig. 2. Electrophoresis of purified 2-deoxy-scyllo-inosose synthase.

A: SDS-PAGE: lane 1; cell-free extract, lane 2; after ammonium sulfate precipitation, lane 3; after DEAE, lane 4; after TSK-gel AF-Blue Toyopearl 650 ML, lane 5; after Superdex 200, lane 6; Mono Q, lane 7; Marker. B: native PAGE.



purified to an electrophoretically homogenous state as described in the Materials and Methods section. The purification procedure is summarized in Table 1. The activity of a cell-free extract and a fraction after ammonium sulfate precipitation were unable to be analyzed quantitatively, because of difficulty in performing HPLC analysis due to heavy impurities. Accordingly, the purification factor and the yield were estimated only after the DEAE chromatography. The molecular weight of DOI synthase was estimated to be 54 kDa on Superdex 200 gel-filtration chromatography.

An enzyme preparation obtained by the Mono Q column chromatography gave two peptide bands of 42 kDa and 23 kDa on the SDS-PAGE (Fig. 2A), but a single band was observed on the native-PAGE (Fig. 2B). Therefore, the native enzyme seemed to be a heterodimeric protein. To confirm this, the enzyme solution after the Mono Q column chromatography was further applied to gel-filtration. Although the peptides of 42 kDa and 23 kDa were expected to be separated by the chromatography if these were not a heterodimeric protein, only a single protein band was eluted under the non-denaturing conditions. On the other hand, these peptides were separated under the denaturing conditions with the same gel column equilibrated with the same buffer (pH 7.7) containing 0.1% SDS. The purified enzyme preparation also gave a sharp single band of protein by the isoelectric focusing chromatography (data not shown). These results strongly suggest that DOI synthase is a heterodimeric enzyme of 65 kDa with peptides of 42 kDa and 23 kDa. The difference between

Table 1. Purification of 2-Deoxy-*scyllo*-inosose synthase from *B. circulans*.

Step	Total protein (mg)	Total activity (U)	Specific activity ^b (U/mg)	Purification factor ^b	Recovery ^b (%)
Extract	5516	n.d.	n.d. ^a	n.d. ^a	n.d. ^a
40~70% sat. (NH ₄) ₂ SO ₄	2431	n.d.	n.d. ^a	n.d. ^a	n.d. ^a
DEAE	817	99.7	0.122	1	100
Blue ^c	119	42.4	0.356	2.87	42.5
Superdex 200	9.787	60.8	6.23	51.1	61.0
Mono Q	0.778	14.1	18.1	148	14.1

1U = 1nmol/minute 2-deoxy-*scyllo*-inosose production. ^aNot determined. ^bCalculated after DEAE chromatography. ^cTSK-gel AF-Blue Toyopearl 650 ML.

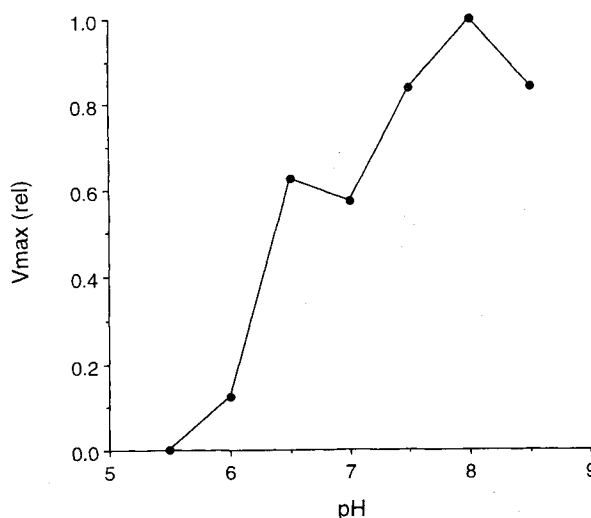
the estimated molecular size 65 kDa by SDS-PAGE and the observed mass 54 kDa determined by Superdex 200 gel-filtration chromatography may be due to a possible tightly packed structure of the native enzyme.

DOI synthase is well-established to be quite similar to dehydroquinase synthase (DHQ synthase) in the shikimate pathway in terms of reaction mechanism^{17,22,23}. DOI synthase, however, seemed to be distinct from the DHQ synthase in the aspect of quaternary structure. As described above, DOI synthase appears to be comprised from a 42 kDa peptide and 23 kDa peptide, whereas DHQ synthases from *E. coli*^{19,20} and *B. subtilis* (*aroB*, SWISS-PROT, P31102) are monomeric enzymes of molecular weight 39 kDa and 41 kDa as determined by the genetic analysis. Interestingly and importantly, the 42 kDa peptide of DOI synthase apparently has a similar molecular weight to those of DHQ synthases. This may indicate that the 42 kDa subunit is responsible for the enzyme reaction with close relationship to DHQ synthases. DHQ synthase purified from *B. subtilis* by HASAN *et al.*²⁹ was reported to be strongly associated with chorismate synthase and flavin reductase. The original purification of DHQ synthase from *E. coli* also suggested possible associated proteins coexisting in the final preparation¹⁹. A possibility that the 23 kDa polypeptide may not be directly related to the reaction catalysis cannot be ruled out at this point. Alternatively, it may be responsible in stabilization or regulatory function.

Enzyme Properties

The effect of pH on the DOI synthase reaction is shown in Fig. 3. The optimum activity was observed between pH 7.5 and 8.5. This characteristic is as same as DHQ synthase²¹. The pH dependence of the reaction indicates the presence of a catalytically important ionization with

Fig. 3. Effect of pH on the enzyme activity.

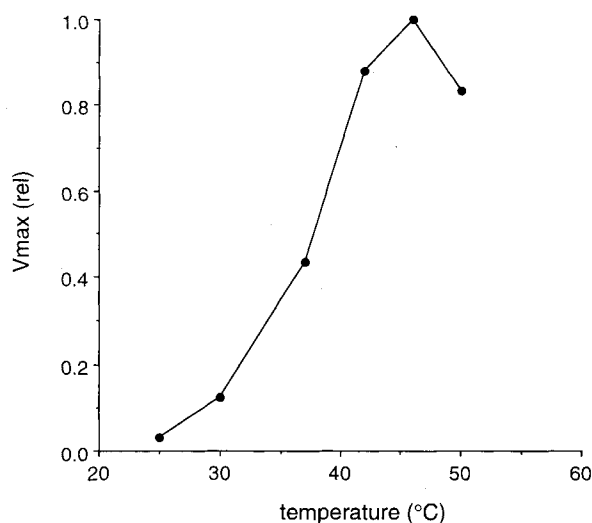


an apparent pKa of about 7.0 as same as DHQ synthase. Thus, this may be an additional support to the postulated similarity between the DOI synthase reaction and the DHQ synthase reaction.

The effect of temperature on the DOI synthase reaction is shown in Fig. 4. An optimum was observed at 46°C under the reaction conditions containing NAD⁺ and Co²⁺. DOI synthase lost all activity at 46°C in the absence of NAD⁺ and Co²⁺ (data not shown). This suggests that NAD⁺ and Co²⁺ are not only used to the enzyme reaction but also stabilize DOI synthase.

The effects of various divalent metal cations on the DOI synthase was studied and the results are shown in Table 2. The presence of metal ion was shown to be essential for the DOI synthase reaction, because no activity was observed in the presence of EDTA. The presence of Co²⁺ ion was most effective to exert the

Fig. 4. Effect of temperature on the enzyme activity.



enzyme activity. The ions of Mg^{2+} , Ca^{2+} , Mn^{2+} were less active, and Cu^{2+} and Zn^{2+} totally diminished the activity. Apparently, DOI synthase reaction requires Co^{2+} ion. In contrast, DHQ synthases from *E. coli*²¹⁾ and *B. subtilis*³⁰⁾ exert its activity in the presence of Co^{2+} or Zn^{2+} , and Co^{2+} or Mn^{2+} respectively. DHQ synthase from *Aspergillus nidulans* exerts its activity in the presence of Co^{2+} or Zn^{2+} , and even Eu^{3+} or Sm^{3+} is capable of activating the DHQ synthase³⁰⁾. According to the crystal structure derived from DHQ synthase from *A. nidulans*, the pentacoordinate Zn^{2+} ion interacts with three amino residues in the active site³¹⁾. It was proposed accordingly that one of the roles of the Zn^{2+} ion was to facilitate the removal of a proton and the hydride transfer. These trends of metal requirement strongly suggest significant difference between DOI synthase and DHQ synthase.

Kinetics of DOI Synthase Reaction

Prior to the kinetic analysis, the time course of the DOI synthase reaction under the optimum conditions with about $1\ \mu M$ of the enzyme was examined first. A rather standard behavior of the reaction was observed, and the reaction rate was decreasing after 2 hours from the initiation. Therefore, a standard protocol was so settled that the enzyme reaction was to be stopped after 1 hour and the production of DOI was quantitated for kinetic analysis.

The kinetic constants (k_{cat} and K_m) were determined by a Lineweaver-Burk plot from the initial velocity of

Table 2. Effect of divalent metal cations on the enzyme activity.

Additive	Relative V _{max}	Additive	Relative V _{max}
None	0.353	Co^{2+}	1
Mg^{2+}	0.398	Ni^{2+}	0.163
Ca^{2+}	0.367	Cu^{2+}	0
Mn^{2+}	0.376	Zn^{2+}	0
Fe^{2+}	0.099	EDTA	0

the DOI synthase reaction with varying the substrate G-6-P concentration, i.e. k_{cat} $7.3 \times 10^{-2} s^{-1}$, K_m (for G-6-P) $9.0 \times 10^{-4} M$, and k_{cat}/K_m $82 M^{-1} s^{-1}$ at $46^\circ C$ and pH 7.7. These values indicate that DOI synthase is a rather inefficient enzyme. It has been shown that, generally, the secondary metabolic enzymes have modest or even poor kinetic properties. The K_m for NAD^+ of DOI synthase was determined to be $1.7 \times 10^{-4} M$ at $46^\circ C$ and pH 7.7.

Comparison of kinetic characteristics between DOI synthase and DHQ synthase seems appropriate at this point. The catalytic activity k_{cat}/K_m of DHQ synthase of *E. coli* is $2.5 \times 10^7 M^{-1} s^{-1}$, and K_m for NAD^+ is $8.0 \times 10^{-10} M$ ($20^\circ C$ and pH 7.75)²¹⁾. DHQ synthase appears to be much more efficient than DOI synthase. This significant difference may be reflected from the difference between primary and secondary metabolic enzymes.

In conclusion, DOI synthase has been purified for the first time to an electrophoretically homogenous state from butirosin-producing *Bacillus circulans* SANK 72073. This may open a way to get more insight into the precise understanding of the biosynthetic enzymes of DOS-containing aminoglycoside antibiotics. DOI synthase was isolated as a heterodimeric enzyme comprising from a 23 kDa- and a 42 kDa polypeptide chains. The actual roles of these two components of DOI synthase have yet to be elucidated. To this end, cloning and overexpression of the genes of each component seem necessary because of a minute availability of DOI synthase from *B. circulans*. In this regard, the chromosomal regions flanking the butirosin resistance gene encoding an aminoglycoside phosphotransferase (*aphA4/butA*) of *B. circulans* was previously investigated by AUBERT-PIVERT and DAVIES to identify the biosynthetic genes³²⁾. The putative gene (*butB*) immediately upstream from *aphA* was not reported to be unrelated to the biosynthesis of butirosin, but rather, was identified

as a gene of the cell-wall-associated protein that eliminates detectable antibiotic accumulation³²). Accordingly, up to now, no biosynthetic genes of the DOS-containing aminoglycoside antibiotics have been found. The present results appear to be crucial, for example, to the future reverse-genetics of the biosynthetic genes of these clinically important class of antibiotics.

Acknowledgments

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