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Stereospecificity of hydride transfer in NAD^+-catalyzed 2-deoxy-*scyllo*-inosose synthase, the key enzyme in the biosynthesis of 2-deoxystreptamine-containing aminocycl itol antibiotics

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Abstract

The key enzyme in the biosynthesis of clinically important aminocyclitol antibiotics is 2-deoxy-*scyllo*-inosose synthase (DOIS), which converts ubiquitous D-glucose 6-phosphate (G-6-P) into the specific carbocycle, 2-deoxy-*scyllo*-inosose with an aid of NAD^+–NADH recycling. The NAD^+-dependent first step of the DOIS reaction was examined in detail by the use of 6-phosphonate and 6-homophosphonate analogs of G-6-P. Both analogs showed competitive inhibition against the DOIS reaction with K_i values of 1.3 and 2.8 mM, respectively, due to their inability for the subsequent phosphate elimination. Based on the direct spectrophotometric observation of NADH formed by the hydride transfer from 6-phosphonate to NAD^+, the stereospecificity of the hydride transfer in the DOIS reaction was analyzed with 6-[4-^{2}H]phosphonate and was found to be pro-*R* specific.

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1. Introduction

2-Deoxy-*scyllo*-inosose synthase (DOIS) catalyzes the conversion of ubiquitous D-glucose 6-phosphate (G-6-P) into a specific six-membered carbocyclic compound, 2-deoxy-*scyllo*-inosose (DOI), in the key step of the biosynthesis of clinically important 2-deoxystreptamine-containing aminocyclitol antibiotics, such as neomycins, kanamycins, paromomycins, ribostamycin, butirocins, gentamicins, etc. [1,2]. We have been involved quite some time in the enzymological studies of the first committed biochemistry in the 2-deoxystreptamine biosynthesis and several key issues have already been addressed. DOIS catalyzes the multi-step and direct cyclization reaction of G-6-P consisting five crucial transformations, which include the initial NAD^+-dependent dehydrogenation at C-4, the elimination of a phosphate from C-5 and C-6, the hydride comeback to C-4 to form a cyclic enol ether, and the finial aldol-type intramolecular condensation between C-6 and C-1 as shown in Fig. 1 [3–5]. The DOIS enzyme was successfully purified from *Bacillus circulans* SANK72073 [6], and the structural gene (*btrC*) for DOIS was identified and heterologously overexpressed in *Escherichia coli* [6,7].

Apparently, DOIS catalyzes the most sophisticated conversion of biologically ubiquitous G-6-P, and the DOI product has been envisioned to provide a useful green chemical source because the six-membered carbocyclic ring structure of DOI can be conveniently converted into important industrial materials such as catechol and other benzenoids [8,9]. To explore the details of this sophisticated enzyme

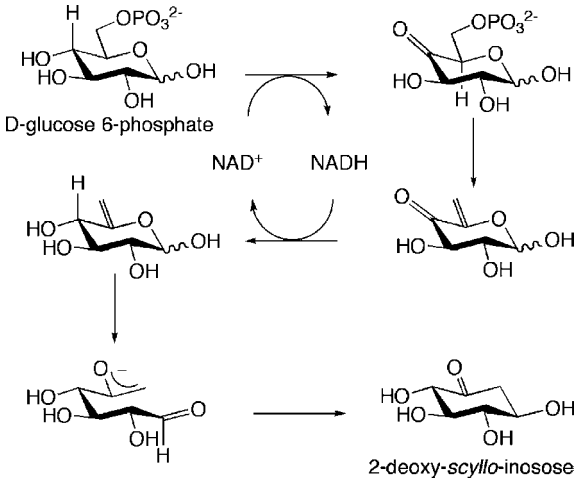
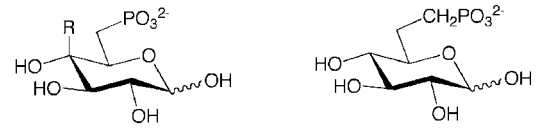


Fig. 1. The reaction mechanism of 2-deoxy-*scyllo*-inosose synthase.



R=H; glucose 6-phosphonate
R=^{2}H; [4-^{2}H]glucose 6-phosphonate

glucose 6-homophosphonate

Fig. 2. Structures of glucose 6-phosphonate, glucose 6-homophosphonate.

reaction and the potential of enzymatic utility, several substrate analogs were tested with the purified BtrC. Direct involvement of the oxygen functionalities at C-1, C-4, C-5, and C-6 in the reaction allowed to envisage the C-2 and C-3 hydroxy groups were relevant for the substrate recognition. In fact, 2-deoxy- and 3-deoxy-D-glucose 6-phosphates were accepted as a substrate and were converted into the corresponding dideoxy-*scyllo*-inosose products, although the conversion was not very effective [10]. The substrate recognition of DOIS was further analyzed using fluorinated and amino analogs. Specific hydrogen-bond accepting and donating interactions between the hydroxy groups at C-2 and C-3 of the substrate and the enzyme were shown to be responsible for the recognition [11]. Furthermore, the cyclization stereochemistry of the DOIS reaction has recently been analyzed by the use of a coupled reaction with hexokinase-ATP and labeled precursors, D-(6*S*) and (6*R*)-[6-$^2H_1$]glucose. As the results, anti elimination of a phosphate and the aldol condensation through a chair conformation has been postulated to the DOIS reaction [12]. More recently, the investigation by the use of a potent mechanism-based and irreversible inhibitor, a carbocyclic analog of G-6-P, demonstrated that Lys-141 of BtrC locates in the vicinity of a phosphate group of G-6-P in the active site and plays a key role for the phosphate recognition and the reaction catalysis [13].

Depicted as above in Fig. 1, the NAD^+ coenzyme apparently functions as a key hydride carrier for both hydrogen abstraction and returning [3–5]. In this paper, we describe the precise analysis of the initial step of the DOIS reaction, including the substrate recognition of DOIS on C-6 phosphate and the stereospecificity of hydride transfer reaction to the nicotinamide ring, by the use of phosphonate and homophosphonate substrate derivatives (Fig. 2).

2. Results and discussion

Phosphonate and homophosphonate analogs of natural phosphate ester, in which a C–O–P array is replaced either by a C–P or a C–CH_2–P group, respectively, are expected to act as either a substrate or a competitive inhibitor for enzymes that catalyze reactions involving a phosphate ester [14]. In the DOIS reaction, the cyclization commences with the hydride transfer from C-4 of G-6-P to NAD^+ as shown in Fig. 1. The α,β-unsaturated carbonyl intermediate is subsequently formed after the facilitated elimination of phosphate at C-6 [13]. Therefore, when the phosphate substituent at C-6 of G-6-P is replaced, for example, by a phosphonate, the elimination could

be obstructed so that the DOIS reaction should be interrupted at this step [15,16]. As a consequence, eventual accumulation of the 4-keto intermediate and the reduced form of coenzyme NADH is anticipated. To obtain direct evidence on the transient formation of NADH in the DOIS NAD^+–NADH recycling system, we attempted to analyze the DOIS reaction by the use of 6-phosphonate and 6-homophosphonate, since these phosphonates appeared to be isoelectronic to the phosphate being essential for the substrate recognition. D-Glucose 6-phosphonate and 6-homophosphonate analogs were prepared according to the literature methods [17,18].

Since both analogs were expected to be an inhibitor for the DOIS reaction as mentioned above, the inhibition assay was first carried out with a purified recombinant DOIS (BtrC derived from butirosin-producing *B. circulans*) [7]. As a result, both of 6-phosphonate and 6-homophosphonate analogs were shown to be moderate competitive inhibitors as expected. The inhibition constants K_i were estimated by plotting the reciprocal of initial rates against inhibitor concentrations. The resulting K_i values (6-phosphonate, 1.3 mM; 6-homophosphonate, 2.8 mM) indicated that the affinity of both analogs to BtrC are not as high as natural substrate G-6-P (K_m, 0.21 mM) and the phosphonate analog has a slightly better affinity than the bulkier homophosphonate. These results appeared to suggest that, while the 6-phosphate group of the substrate G-6-P is important for the substrate recognition, a rather cramped space is provided in the active site of DOIS for the accommodation of the substrate C-6 position. Since the 6-phosphonate analog was more potent than the 6-homophosphonate analog, the former was used for the following experiments.

As described above, the 6-phosphonate analog was anticipated to be involved only in the first dehydrogenation step by being devoid of the elimination step in the DOIS reaction. Thus, the accumulation of NADH was expected for the reaction of this analog with BtrC, and the absorbance at 340 nm during the reaction was monitored. The UV spectrum of the reaction mixture after 5 min incubation is shown in Fig. 3, which clearly indicates that the absorbance at 340 nm was increased by the BtrC reaction with 6-phosphonate. These results confirmed the hydride transfer from 6-phosphonate to NAD^+. To further determine the precise site and

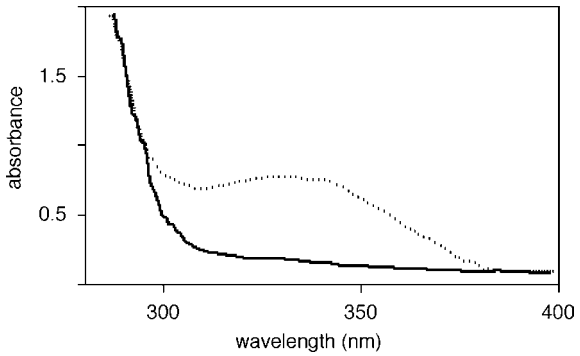


Fig. 3. UV spectra of DOIS reaction with glucose 6-phosphonate after 5 min. Solid line, control; dashed line, DOIS reaction with glucose 6-phosphonate.

stereospecificity of the hydride transfer, we pursued the DOIS reaction by the use of a 6-phosphonate analog with deuterium substitution at C-4.

D-[4-^{2}H]Glucose 6-phosphonate (90% atom ^{2}H by ^{1}H NMR analysis) was similarly synthesized from D-glucose by way of D-[4-^{2}H]glucose [19]. Then, the synthesized D-[4-^{2}H]glucose 6-phosphonate was incubated in a rather large scale with an equimolar NAD^+ and an equimolar BtrC for 30 min at 37 °C. Several efforts for separation of the product NADH from the incubation mixture by ultrafiltration and/or Sephadex G-75 gel filtration [20] were attempted without success. However, after further attempts, denaturation of the enzyme by adding equal amount of sodium dodecyl sulfate (SDS) was found to be effective. After SDS treatment, the mixture was directly charged on Sephadex G-75 chromatography. The fractions containing the product NADH were successfully separated from the denatured enzyme. Further purification by DEAE Sephadex A25 anion exchange chromatography with a linear gradient of NH_4HCO_3 concentration from 5 to 500 mM allowed to obtain purified NADH. The chemical structure of this product was carefully confirmed by ^{1}H NMR spectroscopy (Fig. 4) to the [4-^{2}H] labeled NADH. Apparently, the deuterium at the C-4 position of the 6-phosphonate analog was transferred specifically to the pro-*R* position at C-4 in the nicotinamide ring of NADH coenzyme [21] as shown in Fig. 5. Unfortunately, the intermediate 4-keto derivative of 6-phosphonate was unable to be isolated probably due to its instability under these purification conditions.

As was previously reported, the reaction mechanism of DOIS was pointed out to be similar to that of dehydroquinate synthase (DHQS) catalyzing the cyclization of 3-deoxy-D-*arabino*-heputulosonate-7-phosphate to dehydroquinate in the shikimate pathway of primary metabolism [3–5]. Moreover, DOIS (BtrC) was found to be similar to DHQS to a certain extent (25–35%) to the amino acid level [7]. The three-dimensional structure of DHQS complexed with NAD^+ and a phosphonate substrate analog from *Emericella nidulans* was recently elucidated by X-ray crystallography [22], which suggested that DHQS has the pro-*R* specificity of the

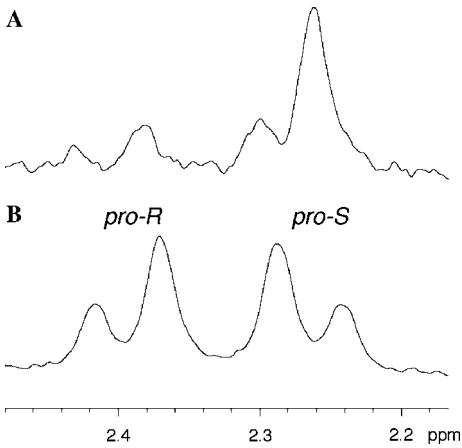


Fig. 4. Partial ^{1}H NMR spectra (400 MHz, D_2O) of (A) deuterated NADH from [4-^{2}H]glucose 6-phosphonate with DOIS; (B) Non-labeled NADH. Signals were assigned according to Arnold et al. [21].

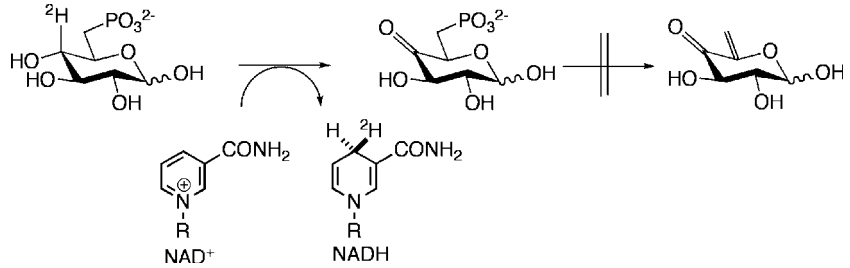


Fig. 5. DOIS reaction with [4-^{2}H]glucose 6-phosphonate.

hydride transfer as does DOIS. Apparently, DOIS (BtrC) clearly belongs to the family of DHQS. However, dissimilarity between DOIS and DHQS was also found, particularly in the stereochemistry of overall reactions [12]. It may thus be pointed out that the evolutionary aspect of DOIS involved in microbial secondary metabolism and DHQS in a primary metabolism are intriguing.

In conclusion, the present study clearly demonstrated that the 6-phosphonate and 6-homophosphate analogs of G-6-P were accepted by 2-deoxy-*scyllo*-inosose synthase and behaved as a competitive inhibitor. Further, among the multi-step reactions, only the initial hydride transfer reaction took place with the 6-phosphonate analog, and the DOIS reaction was arrested at this stage. By the use of deuterated 6-phosphonate analog, we successfully demonstrated the direct evidence for the hydride transfer and its stereospecificity. The hydride transfer in the DOIS reaction mediated by NAD coenzyme was found to be pro-*R* stereospecific.

3. Materials and method

3.1. General information

6-Phosphonate and 6-homophophonate analogs were prepared basically according to the known methods [17,18]. Purified DOIS (BtrC) was prepared according to the method of Kudo et al. [7]. Protein concentration was quantitated by the Lowry–Folin method with bovine serum albumin as standard. Sephadex G75 and DEAE Sephadex A25 were purchased from Amersham Biosciences and Sigma–Aldrich, respectively. Deuterium oxide (99.8% atom enriched, Merck) was used for NMR solvent. NMR chemical shifts were reported in δ value based on HDO ($\delta_H = 4.65$) and dioxane ($\delta_C = 67.4$).

3.2. D-[4-^{2}H]Glucose 6-phosphonate

^{1}H NMR (400 MHz, D_2O) δ 5.02 (d, $J = 4.1$ Hz, H-1, α-anomer), 4.47 (d, $J = 8.0$ Hz, H-1, β-anomer), 3.90 (m, H-5, α-anomer), 3.50 (d, $J = 9.7$ Hz, H-3, α-anomer), 3.47 (m, H-5, β-anomer), 3.36 (dd, $J = 3.8$, 9.7 Hz, H-2, α-anomer), 3.28 (d, $J = 9.2$ Hz, H-3, β-anomer), 3.07 (dd, $J = 8.0$, 9.5 Hz, H-2, β-anomer), 2.19 (ddd,

$J = 2.6$, 15.8, 16.3 Hz, H-6, α-anomer), 2.14 (ddd, $J = 2.6$, 15.8, 16.3 Hz, H-6, β-anomer), 1.82 (ddd, $J = 5.6$, 15.6, 15.8 Hz, H-6, α-anomer), and 1.76 (ddd, $J = 5.6$, 15.6, 15.8 Hz, H-6, β-anomer); ^{13}C-NMR (99.5 MHz, D_2O): δ 96.4, 92.4, 75.8, 74.6, 73.4 (t, $J = 20$ Hz), 72.9, 71.9, 71,1 (t, $J = 20$ Hz), 67.4, 67.3, 29.8 (d, $J = 140$ Hz), and 29.9 (d, $J = 140$ Hz). Anal. Calcd. for $C_6H_{12}{}^2HO_8P$: C, 29.42; H+^{2}H, 5.68; Found: C, 29.33; H+^{2}H, 5.85.

3.3. Inhibition assay

A solution of purified BtrC (50 μl, 2 μM) was added to a mixture of various amount of 6-phosphonate or 6-homophosphonate (0–50 mM) with NAD^+ (5 mM), G-6-P (5 mM), and $CoCl_2$ (0.2 mM), in Tris–HCl buffer (pH 7.7, 100 μl). Incubation was carried out at 46 °C for 5 min and the reaction was terminated by adding 100 μl of methanol. After adding 20 μl of *O*-(4-nitrobenzyl)-hydroxylamine hydrochloride in pyridine (5 mg/ml), the resulting mixture was heated at 60 °C for 1 h, and then the solvent was removed by flushing the air. The residue was analyzed by a HPLC method as described previously to quantitate the amount of the reaction products [5]. The inhibition kinetics data were graphically analyzed by Lineweaver–Burk double reciprocal plots.

3.4. Isolation of the labeled NADH

A solution of purified BtrC (10 ml, 1 mM) was added to a mixture of 6-[4-^{2}H]phosphonate (6 mg, 2 mM), NAD^+ (14 mg, 2 mM), and $CoCl_2$ (0.2 mM) in a Tris–HCl buffer (pH 7.7, 10 ml). Incubation was carried out at 37 °C for 30 min and the reaction was terminated by adding SDS (10 mg). The mixture was stirred at room temperature for 5 min, and then directly charged on a Sephadex G75 gel column (1.5 cm i.d. × 45 cm). The column was adjusted to pH 8.0 using 25 mM NH_4HCO_3. Absorbance was monitored at 260, 280, and 340 nm for each fraction. Fractions having A_{260}/A_{340} ratio of approximately 23.0, which containing NAD^+ and NADH mixture, were collected. Separation of NAD^+ and NADH was performed by a DEAE Sephadex A25 anion exchange chromatography (1.5 cm i.d. × 5 cm) using a linear gradient from 5 to 500 mM NH_4HCO_3, pH 8.0. The fractions containing NADH were collected and concentrated to dryness.

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References

[1] K.L. Rinehart Jr., R.M. Stroshane, R. M, J. Antibiot. 29 (1976) 319–353.

[2] K.L. Rinehart Jr., T. Suami (Eds.), Aminocyclitol Antibiotics, ACS Symposium Series No. 125. American Chemical Society, Washington DC, 1980.
[3] K. Kakinuma, Y. Ogawa, T. Sasaki, H. Seto, N. Otake, J. Am. Chem. Soc. 103 (1981) 5614–5616.
[4] K. Kakinuma, Y. Ogawa, T. Sasaki, H. Seto, N. Otake, J. Antibiot. 42 (1989) 926–933.
[5] N. Yamauchi, K. Kakinuma, J. Org. Chem. 60 (1995) 5614.
[6] F. Kudo, Y. Hosomi, H. Tamegai, K. Kakinuma, J. Antibiot. 52 (1999) 81–88.
[7] F. Kudo, H. Tamegai, T. Fujiwara, U. Tagami, K. Hirayama, K. Kakinuma, J. Antibiot. 52 (1999) 559–571.
[8] K. Kakinuma, E. Nango, F. Kudo, Y. Matsushima, T. Eguchi, Tetrahedron Lett. 41 (2000) 1935–1938.
[9] C.A. Hansen, J.W. Frost, J. Am. Chem. Soc. 124 (2002) 5926–5927.
[10] N. Iwase, F. Kudo, N. Yamauchi, K. Kakinuma, Biosci. Biotechnol. Biochem. 62 (1998) 2396–2407.
[11] T. Eguchi, S. Sasaki, Z. Huang, K. Kakinuma, J. Org. Chem. 67 (2002) 3979–3984.
[12] E. Nango, F. Kudo, T. Eguchi, K. Kakinuma, Chem. Lett. 32 (2003) 438–439.
[13] E. Nango, T. Eguchi, K. Kakinuma, J. Org. Chem. 69 (2004) 593–600.
[14] C. Schultz, Bioorg. Med. Chem. 11 (2003) 885–898.
[15] S.L. Bender, T. Widlanski, J.R. Knowles, Biochemistry 28 (1989) 7560–7572.
[16] T. Widlanski, S.L. Bender, J.R. Knowles, Biochemistry 28 (1989) 7572–7582.
[17] N.S. Padyukova, S. Afsar, H.B.F. Dixon, M.Y. Karpeiskii, Bioorganicheskaya Khimiya 21 (1995) 382–385.
[18] D.J.W. Roach, R. Harrison, C.R. Hall, T.D. Inch, Biochem. J. 197 (1981) 735–737.
[19] R.G.S. Ritchie, N. Cyr, B. Korsch, H.J. Koch, A.S. Perlin, Can. J. Chem. 53 (1975) 1424–1433.
[20] S.F. Wang, O. Gabriel, J. Biol. Chem. 245 (1970) 8–14.
[21] L.J. Arnold Jr., K-.S. You, A.S. William, N.O. Kaplan, Biochemistry 15 (1976) 4844–4849.
[22] E.P. Carpenter, A.R. Hawkins, J.W. Frost, K.A. Brown, Nature 395 (1998) 299–302.