BIOCHEMICAL STUDIES ON 2-DEOXY-*SCYLLO*-INOSOSE, AN EARLY INTERMEDIATE IN THE BIOSYNTHESIS OF 2-DEOXYSTREPTAMINE

IV. A CLUE TO THE SIMILARITY OF 2-DEOXY-*SCYLLO*-INOSOSE SYNTHASE TO DEHYDROQUINATE SYNTHASE

Sir:

2-Deoxystreptamine (**1**), is a common aminocyclitol aglycon in a major group of clinically important aminoglycoside antibiotics. The crucial step in the biosynthesis of **1** is the formation of the precursor, 2-deoxy-*scyllo*-inosose (**2**), from D-glucose (**3**) *via* the intramolecular C-C bond formation between C-1 and C-6.[1,2] The transformation of **3** into **2** was proposed by us to involve a multi-step mechanism as shown in Scheme 1, the chemistry of which was suggested to be similar to the dehydroquinate synthase in the shikimate pathway,[3,4] and an enzyme responsible of the intramolecular cyclization was named "2-deoxy-*scyllo*-inosose synthase"[5~8] AKHTAR then reported that the C-4 hydrogen of **3** was lost during the biosynthesis of **1** in the whole cells of *Streptomyces fradiae* (producing neomycins) and suggested involvement of oxidoreduction at C-4 of the substrate.[7,8] However, nothing has so far been clarified as to whether a single enzyme is involved or certain dissociable enzymes cooperate to form **2**. This communication is an approach to this problem.

Recently, we established a cell-free system from *Streptomyces fradiae* IFO 13147 cells and successfully observed the production of **2** from D-glucose-6-phosphate (**4**) in the presence of NAD.[9] Using this system, we studied the closer insight into the reaction mechanism of the aforementioned 2-deoxy-*scyllo*-inosose synthase, focusing on the fate of the C-4 hydrogen of **4**.

A partially purified enzyme was prepared from the $(NH_4)_2SO_4$ saturation precipitate derived from the $10,000 \times g$ supernatant of the sonicate of *S. fradiae* cells.[9] After dialysis of the precipitate, the enzyme fraction (20 ml, 15 mg protein/ml) was chromatographed over a DEAE-Cellulofine A-800 column (i.d. 1 cm × 15 cm, buffer: 50 mM Tris-HCl, pH 7.5, containing 0.2 mM of Co^{2+} and Mg^{2+}) with a linear gradient of the NaCl (0 to 0.4 M) concentration. The enzyme activity was assayed by the HPLC method described previously.[9] Appropriate enzyme fractions were collected and used for the isotope-tracer experiments.

D-[4-^{2}H]-**3** (>95% enriched), which had been synthesized by a literature-cited procedure with slight modifications,[10] was chemically converted to D-[4-^{2}H]-**4**. The enzyme reaction was carried out at 37°C for 2 hours with a mixture of the enzyme fraction, 2 mM of NAD and 1 mM of either the labeled or the non-labeled substrate. D-[6,6-$^2H_2$]-**4** was used as a positive reference.[5,6] Production of **2** was quantitated by the aforementioned HPLC analysis.[9] The yields usually ranged around 10%. No endogenous or residual **2** was observed in the control experiment. Significant reduction (~40%) of the production of **2** was observed in the experiments with the D-[4-^{2}H]-**4** substrate compared with the non-labeled or D-[6,6-$^2H_2$]-**4** substrate (duplicate, data not shown). These observations may be suggestive of the primary kinetic isotope effect

Scheme 1. The proposed mechanism of 2-deoxy-*scyllo*-inosose (**2**) synthase reaction in the biosynthesis of 2-deoxystreptamine (**1**).

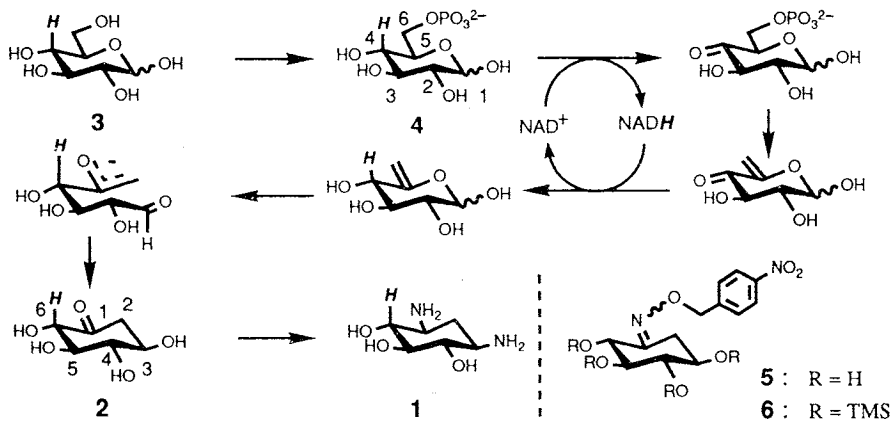


Table 1. Relative intensities of the mass spectra of **6** obtained from the enzyme reaction products and synthetic standard[a].

Substrate	D-Glucose-6-phosphate		D-[4-^{2}H]Glucose-6-phosphate		Chemically synthesized [6-^{2}H]-2-deoxy-*scyllo*-inosose
	Run 1	Run 2	Run 1	Run 2	
m/z 599	0	0	0	0	0
600	100	100	56.6	57.3	0
601	50.3	49.0	100	100	100
602	26.3	26.4	50.6	52.4	48.3
603	10.5	9.1	26.3	25.3	28.9

[a] Mass spectral scanning was performed in triplicate for each case.

in the oxidoreduction during the 2-deoxy-*scyllo*-inosose synthase reaction.

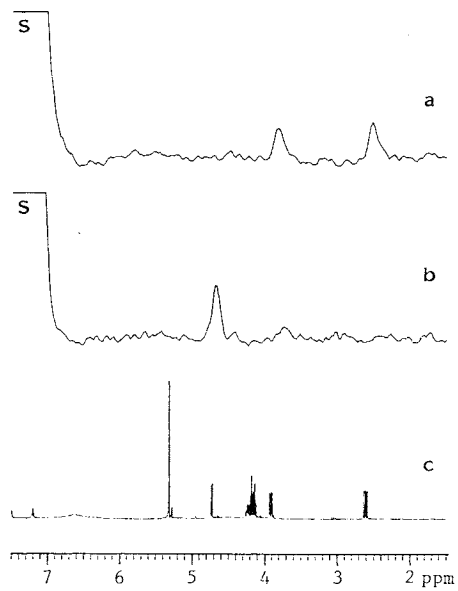
The deuterium enrichment of the enzyme reaction product was determined by mass spectrometry. The *O*-(4-nitrobenzyl)oxime derivatives **5**, prepared from each enzyme reaction, were further separately silylated to the corresponding trimethylsilyl (TMS) ethers **6**. Each reaction mixture was diluted, and then subjected to GC-MS (Shimadzu-LKB 9020 DF spectrometer; OV-1, 12 m).

The relative signal intensities of the molecular ion region of **6** obtained from each enzyme reaction are shown in Table 1. The non-labeled control showed the M^+ ion at *m/z* 600, but nothing at *m/z* 599, and only the M^+ ion (*m/z* 601) was observed in the spectrum of chemically synthesized [6-^{2}H]-**5** from D-[4-^{2}H]-**3** as well.[11] In contrast, the enzyme reaction product from D-[4-^{2}H]-**4** showed the ions at *m/z* 600 and 601 (M^+), suggesting the formation of both non-labeled and monodeuterated-**2**. Calculation allowed us to estimate the deuterium content in the product from the D-[4-^{2}H]-**4** substrate to be 56%. Based upon the previously proposed reaction mechanism, the deuterium of D-[4-^{2}H]-**4** was expected to be incorporated into C-6 of **2**, which has ultimately been proved as follows.

The location of deuterium in **5** obtained from the D-[4-^{2}H]-**4** substrate was determined unambiguously by ^{2}H NMR spectra as shown in Fig. 1. The ^{2}H chemical shifts were unequivocally assigned by comparison with the corresponding ^{1}H NMR spectrum of the non-labeled oxime **5**. A positive control, [2,2-$^2H_2$]-**3** formed enzymatically from D-[6,6-$^2H_2$]-**4**, clearly showed the signals at δ 2.54 and 3.81 to be due to the C-2 methylene group (^{1}H NMR, δ 2.58 and 3.89).[5,6] Most crucial is that the derivative **5** obtained from D-[4-^{2}H]-**4** showed a single deuterium signal at δ 4.65, which was attributed to the C-6 position (^{1}H NMR, δ 4.71). Thus, the deuterium of the D-[4-^{2}H]-**4** substrate

Fig. 1. ^{2}H NMR spectra (73.85 MHz, C_5H_5N) of **5** from enzyme reaction products and ^{1}H NMR spectrum (500 MHz, C_5D_5N) of the non-labeled standard.

a: (^{2}H) the product from D-[6,6-$^2H_2$]glucose-6-phosphate; b: (^{2}H) the product from D-[4-^{2}H]glucose-6-phosphate; c: (^{1}H) non-labeled standard. "S" is the natural abundance signal of deuterium in the solvent.



appears to be incorporated into the C-6 position of **2**.

Of significance is that the deuterium at the C-4 position of D-[4-^{2}H]-**4** is retained in the product during the *in vitro* reaction of the NAD-assisted 2-deoxy-*scyllo*-inosose synthase reaction. Thus, the deuterium may be held in the vicinity of the substrate during the NAD-assisted oxidation and reduction. The observed enrichment ratio can be explained as follows. The non-deuterated **2** was probably derived from a minute amount of non-labeled **4** in the D-[4-^{2}H]-**4** specimen. Accordingly, the low transfor-

mation efficiency (~10% chemical yield) and a possible kinetic isotope effect in the oxidoreduction at C-4 seem to effect preferential conversion of the non-labeled substrate, resulting in significant formation of the non-labeled **2**. The aforementioned AKHTAR's *in vivo* results can be explained similarly[7,8]

From the present *in vitro* experiments using a partially purified enzyme, it appears that the formation of **2** from **4** indeed requires NAD and the hydrogen at C-4 of the substrate is retained in the product, thereby suggesting that a series of reactions of 2-deoxy-*scyllo*-inosose formation may be performed by a single enzyme, "2-deoxy-*scyllo*-inosose synthase", with catalytic turn over of the NAD cofactor.

As to the mechanism of dehydroquinate synthase with respect to the 2-deoxy-*scyllo*-inosose synthase reaction, two features may be emphasized: 1) The C-5 hydrogen (at the β-position to the phosphate group, synonymous to the C-4 hydrogen of **4**) of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate is retained in the cyclization reaction,[12] and 2) the NAD requirement is only catalytic and NAD is tightly bound to the enzyme.[3] While it is not clear that the 2-deoxy-*scyllo*-inosose synthase catalyzes only the oxidoreduction and/or cyclization reaction, as has been discussed for the dehydroquinate synthase,[4] the present results suggest close similarity of 2-deoxy-*scyllo*-inosose synthase, functioning in microbial "secondary metabolism", to dehydroquinate synthase in respect to the mechanism. The more datailed comparison must await closer analysis of the former enzyme.

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