Stereochemistry of an $\alpha,\beta$-elimination reaction by D-glucosaminate dehydratase

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The proton NMR analysis of D-glucosaminate dehydratase reaction in D$_2$O revealed the incorporation of a deuterium atom at C-3 carbon of the product, 2-keto-3-deoxy-D-gluconate. Based on the chemical shift of C-3 proton of the product and the coupling constant characteristic for the C-3 and C-4 axial-axial coupling in the $\delta$-C$_5$ pyranose conformation, the deuterium is in the pro-S position. Thus, the dehydration of D-glucosaminate by the enzyme proceeds in a retention mode at C-3 carbon. Kinetic parameters show that the rate-determining step is the abstraction of $\alpha$-proton from the substrate.

1. INTRODUCTION

D-Glucosaminate dehydratase (EC 4.2.1.26), a pyridoxal 5'-phosphate (pyridoxal-P) enzyme, catalyzes the $\alpha,\beta$-elimination reaction of D-glucosaminate to yield 2-keto-3-deoxy-D-gluconate and ammonia.

Stereochemical studies of $\alpha,\beta$-elimination reactions catalyzed by a variety of pyridoxal-P enzymes such as D-serine dehydratase [1], L-threonine dehydratase [2], D-threonine dehydratase [2,3], S-alkylcysteine lyase [4], tyrosine phenol-lyase [5], tryptophanase [6,7] and $\beta_2$ subunit of tryptophan synthetase [8], have provided a common feature of the mechanism of pyridoxal-P-dependent $\alpha,\beta$-elimination reactions as reviewed in [9]. The reactions catalyzed by these enzymes proceed exclusively with retention of configuration at C-3 carbon of substrates; the substitution by a new group (a proton) occurs from the same direction as the leaving group is abstracted. We here describe that D-glucosaminate dehydratase, the only pyridoxal-P enzyme that acts specifically on an amino sugar derivative, also carries out the stereo-retentive incorporation of a deuterium from solvent at the C-3 position of 2-keto-3-deoxy-D-gluconate during the dehydration of D-glucosaminate in D$_2$O.

2. MATERIALS AND METHODS

D-Glucosaminate and pyridoxal-P were obtained from Nakarai Chemicals (Kyoto). Deuterium oxide (99.7%) was obtained from Merck. The deuterated potassium phosphate buffer (0.2 M)
was prepared by dissolving appropriate quantities of K₂HPO₄ and KH₂PO₄ in D₂O and adjusting to pD 8.4. D-Glucosaminate dehydratase was purified from Agrobacterium radiobacter as in [10]. All other chemicals were analytical grade reagents. Proton NMR-spectra were taken with a Jeol JNM-NM-100 spectrometer. Chemical shifts are reported as ppm downfield from sodium 3-trimethylsilyl-2-[2,2,3,3-²H]propionate. The enzyme was assayed by determination of 2-keto-3-deoxy-D-gluconate formed with semicarbazide [10]. Thin-layer chromatography (TLC) was done on a Merck silica gel plate, no. 5553, with a solvent system, n-butanol–pyridine–water (1:1:1, by vol.), detecting with H₂SO₄.

The enzyme reaction was carried out in D₂O at 37°C as follows. D-Glucosaminate (51.3 µmol) and pyridoxal-P (0.1 µmol) were dissolved in 0.45 ml of the deuterated buffer. The reaction was initiated by addition of 2 units of enzyme. NMR-spectra were taken at time intervals.

3. RESULTS AND DISCUSSION

3.1. Proton NMR-spectra of the authentic D-glucosaminate and 2-keto-3-deoxy-D-gluconate

Proton NMR-spectra of the authentic D-glucosaminate and 2-keto-3-deoxy-D-gluconate in D₂O showed that α-proton of D-glucosaminate, δ 4.4 ppm (1 H, d), and β-protons of 2-keto-3-deoxy-D-gluconate, δ 2.2–2.4 ppm (1 H, m, 3α-H) and δ 2.5–2.9 ppm (1 H, m, 3eq-H) exhibit characteristic NMR signals for the compounds (fig.1A,D).

![Fig. 1. Proton NMR-spectral change observed during incubation of D-glucosaminate with D-glucosaminate dehydratase in D₂O. The reaction was carried out as described in the text. The spectra were taken at 0 h (A), 3 h (B) and 24 h (C) of the reaction; (D) the authentic 2-keto-3-deoxy-D-gluconate, 10 mg/ml D₂O.](attachment:image)

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3.2. Time course of the enzyme reaction

The enzyme reaction was performed, and a decrease in α-proton signals of the substrate and an increase in β-proton ones of the product were followed. Plots of the integration values of these signals against time took on one curve (fig. 2). This indicates that only one deuterium atom is incorporated at β-position of the product, and the rate determining step of the reaction is the abstraction of α-proton from the substrate.

3.3. Characterization of the reaction product

After incubation for 24 h, α-proton signals of the substrate disappeared. The product was examined by the semicarbazide method and TLC showed that D-glucosaminate was fully converted into 2-keto-3-deoxy-D-gluconate. In contrast to the authentic 2-keto-3-deoxy-D-gluconate, the enzyme reaction product shows only 3α-H signals of β-proton in NMR spectrum (fig. 1C). Thus, a deuterium atom is enzymatically incorporated at 3α-H position of the product from solvent. In addition, the coupling constant of C-3 and C-4 proton signals (d, J = 11 Hz) is in good agreement with the value (J = 12 Hz) reported for C-3 and C-4 axial–axial coupling of methyl(methyl 3-deoxy-β-D-erythro-2-hexulopyranoside)onate in 2C₅ conformation [11]. Thus, the deuterium is unambiguously in the C-3 pro-S position of 2-keto-3-deoxy-D-gluconate (fig. 3). Since the C-3 carbon of D-glucosaminate is in the R configuration, the deuterium is added to the product from the same direction as the hydroxyl group is liberated; the reaction proceeds with a retention mode.

3.4. Isotope effect on D-glucosaminate dehydratase

Ratios of kinetic parameters obtained for the enzyme reaction in D₂O and H₂O were Kₘ(D):Kₘ(H) = 1 and Vₘₐₓ(D):Vₘₐₓ(H) = 0.7–0.8; the deuterium solvent isotope effect on the reaction rate is very small. These results also show that the rate-determining step does not involve the protonation to the C-3 carbon of the product, but involves the abstraction of the α-proton from the substrate.
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