Stereochemistry of an α,β-elimination reaction by D-glucosaminate dehydratase

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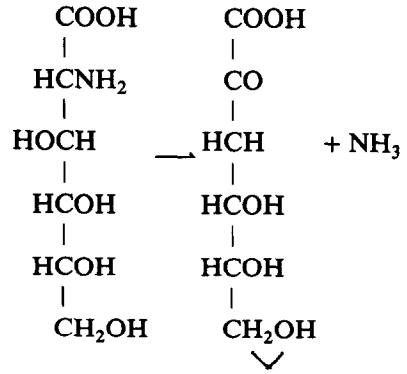
The proton NMR analysis of D-glucosaminate dehydratase reaction in D_2O revealed the incorporation of a deuterium atom at C-3 carbon of the product, 2-keto-3-deoxy-D-gluconate. Based on the chemical shift of C-3 proton of the product and the coupling constant characteristic for the C-3 and C-4 axial–axial coupling in the $^2C_5$ 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 α-proton from the substrate.

D-Glucosaminate dehydratase *α,β-Elimination reaction* *Pyridoxal 5'-phosphate* *Proton NMR analysis* *Stereochemistry* Agrobacterium radiobacter

1. INTRODUCTION

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



Stereochemical studies of α,β-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 β_2 subunit of tryptophan synthetase [8], have provided a common feature of the mechanism of pyridoxal-P-dependent α,β-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_2O.

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)

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was prepared by dissolving appropriate quantities of K_2HPO_4 and KH_2PO_4 in D_2O 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-^{2}H]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 H_2SO_4.

The enzyme reaction was carried out in D_2O 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 D_2O 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*, 3_{ax}-H) and δ 2.5–2.9 ppm (1 H, *m*, 3_{eq}-H) exhibit characteristic NMR signals for the compounds (fig.1A,D).

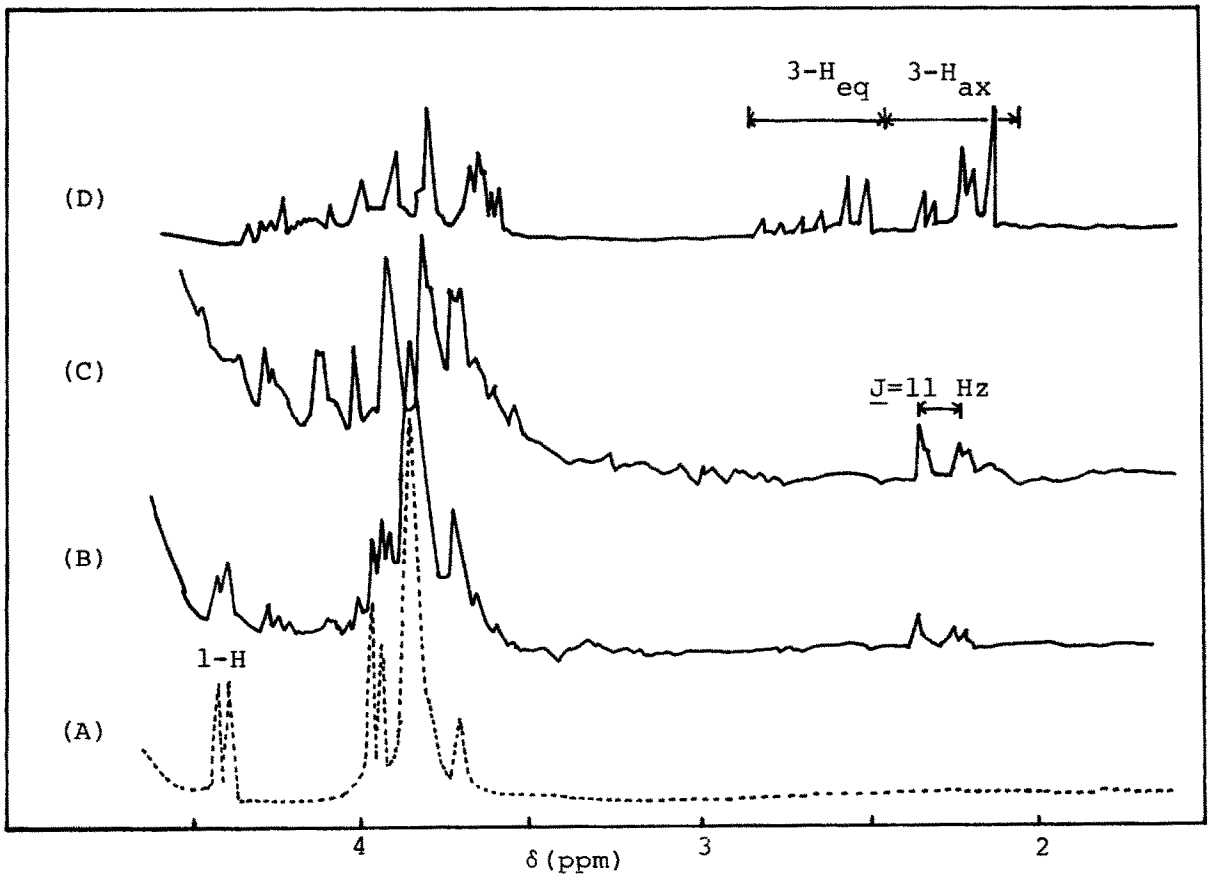

Fig.1. Proton NMR-spectral change observed during incubation of D-glucosaminate with D-glucosaminate dehydratase in D_2O. The reaction was carried out as described in the text. The spectra were taken at 0 h (A), 3 h (B) and 24 h (C) of the reaction; (D) the authentic 2-keto-3-deoxy-D-gluconate, 10 mg/ml D_2O.

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 3_{ax}-H signals of β-proton in NMR spectrum (fig.1C). Thus, a deuterium atom is enzymatically incorporated at 3_{eq}-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

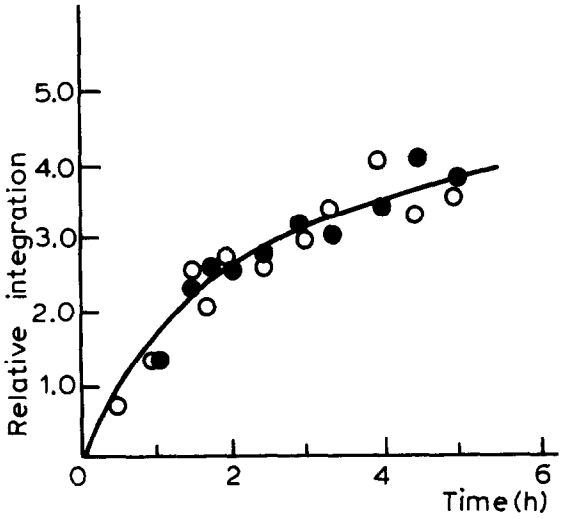

Fig.2. Time course of disappearance of α-proton signals of D-glucosaminate (●) and appearance of β-proton signals of 2-keto-3-deoxy-D-gluconate (○). The disappearance of α-proton of D-glucosaminate was determined by subtraction of the integration value at each incubation time from the initial value (the integration value was 16 under the conditions employed). The integration value of β-proton of 2-keto-3-deoxy-D-gluconate was also plotted against time.

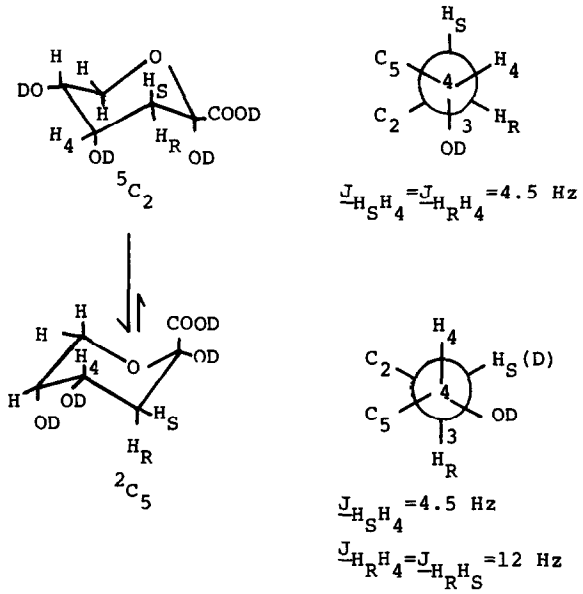

Fig.3. A possible structure of 2-keto-3-deoxy-D-gluconate. 2-Keto-3-deoxy-D-gluconate prefers the $^2C_5$ pyranose form (bottom), because the $^5C_2$ form (top) suffers a 1,3 diaxial effect between substituents at C-2 and C-4. Newman projection diagrams are drawn with respect to the C-3 and C-4 axis. The coupling constant observed (J = 11 Hz) corresponds to $J_{3,4\ ax}$ in the $^2C_5$ conformation.

C-4 axial–axial coupling of methyl(methyl 3-deoxy-β-D-erythro-2-hexulopyranoside)onate in $^2C_5$ 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 D_2O and H_2O were $K_m(D):K_m(H)$ = 1 and $V_{max}(D):V_{max}(H)$ = 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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