MECHANISM OF CELLOBIOSE EPIMERASE[1]

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The mechanism of the epimerization of cellobiose to 4-O-β-D-glucopyranosyl-D-mannose by enzyme preparations from Ruminococcus albus was investigated in deuterium oxide. Only the proton of carbon-two of the reducing moiety is exchanged during the conversion. Cellobiulose is not isomerized by this system. A proposed mechanism involves a carbanion-type but no keto intermediate in this unique epimerization.

EXPERIMENTAL

Enzyme preparation. Ruminococcus albus strain 7 was maintained and grown for enzyme preparation according to the methods in the previous report (1). The enzyme from 305 ml of culture was precipitated by means of 70% saturated ammonium sulfate, collected by centrifugation and redissolved in D_2O and lyophilized.

Epimerization of cellobiose in D_2O. Cellobiose was dissolved in tris-maleate in D_2O, pH 7.0, and lyophilized. The total enzyme and lyophilized substrate were redissolved in D_2O to give a final concentration of 1.0 g cellobiose in 10 ml of 0.06 M tris-maleate. The reaction was flushed with N_2 and incubated at 29°C for 60 hr in the presence of toluene. The resulting disaccharides were adsorbed on a column (2 x 12 cm) of 50% Darco G-60 charcoal and 50% celite 545 (John Mansville Co.). The column was rinsed with water and the disaccharides were eluted with 15% ($\frac{v}{v}$) ethanol.

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Isolation of mannose. The disaccharides were hydrolyzed with 2 N H_2SO_4 for 1 hr at 100°C and subsequently neutralized to pH 7 with barium hydroxide. In order to separate the mannose from glucose, the filtrate was treated with phenylhydrazine at 0°C, with the pH adjusted to 5 with sodium acetate (2). Nearly colorless crystals of mannose phenylhydrazone precipitated on standing overnight, yield 81 mg, m.p. 193-195°C with decomposition. D-Mannose was regenerated from its phenylhydrazone with benzaldehyde (2). The mannose was free of any glucose based on paper chromatography. Both the enzymatically produced mannose and a mannose standard (Fisher Scientific Co.) were dissolved in D_2O, lyophilized and redissolved in D_2O. NMR spectra were determined on each by means of a Varian Model HA-100 NMR spectrometer using sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) as an internal reference signal.

Preparation of cellobiulose. Cellobiulose was prepared by means of alkaline isomerization of cellobiose in saturated $Ca(OH)_2$ at 38°C for 20 hr (3). It was isolated from the reaction mixture by adsorbing to a charcoal-celite column (4.5 x 30 cm) and gradiently eluting the sugars with water to 5% ($\frac{v}{v}$) ethanol. The optical rotation of the isolated cellobiulose was $[\alpha]_D^{25}$ -60° (0.4% in water) in agreement with the value reported by Corbett and Kenner (3). Gas chromatography of the trimethylsilyl derivative on an 8 ft column of 3% OV-17 on Chromosorb W (Applied Science Laboratories) at 250°C resulted in two not completely resolved peaks with 5.8 min and 6.1 min retention time. There was no evidence of cellobiose or glucosylmannose in the cellobiulose based on the gas chromatography data. The NMR spectrum of the cellobiulose showed no anomeric hydrogens present and hence no aldehydes. Cellobiulose was chromatographed on paper with n-propanol:ethyl acetate:water (7:1:2) as a developing solvent system and was detected with dimedon (4). Only one ketose was indicated.

RESULTS AND DISCUSSION

D-Mannose, obtained from enzymatically produced 4-O-β-D-glucopyranosyl-D-mannose in the presence of D_2O, contained a deuterium atom on carbon-two.

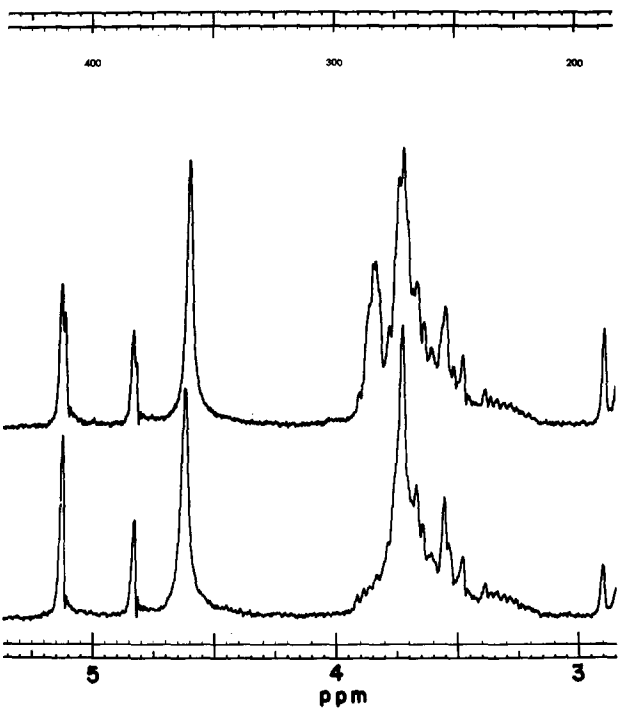

Figure 1. NMR spectra of mannose standard (top) and mannose (bottom) from enzymatic epimerization of cellobiose to 4-O-β-D-glucopyranosyl-D-mannose in D_2O. Spectra were determined at 100 MHz in D_2O solution with internal DSS. Sweep width 500 Hz, offset 100 Hz.

The NMR spectrum (Fig. 1) shows a signal missing in the region of 3.84 to 3.94 ppm when compared with standard mannose. Chemical shifts due to H_2^{α} and H_2^{β} in mannose have been determined previously by spin decoupling experiments and by synthesis of specifically deuterated compounds (5). The resonance signals of H_2^{α} and H_2^{β} on carbon-two of D-mannose are in the region of the missing signal. The rest of the spectrum is characteristic of D-mannose. However, the signals of the anomeric hydrogens at carbon-one of the enzymatically produced mannose are much sharper than those of the anomeric hydrogens of the standard mannose which are partially resolved multiplets. This is consistent with the absence of a vicinal hydrogen (at C-2) which would be expected to split the signal. These splittings largely are dependent on

the conformation at carbon-two (6). These results from the NMR spectra show that the hydrogen on carbon-two of the reducing moiety is exchanged with the solvent system during the epimerization of cellobiose to glucosylmannose.

Isomerization reactions involving carbon-two of monosaccharides usually involve an aldose to ketose conversion (7). Hence, the keto-disaccharide, cellobiulose, was prepared and tested as a possible intermediate in the enzymatic conversion. However, cellobiulose showed no reaction with the enzyme while the control reaction with cellobiose showed considerable epimerization to glucosylmannose. The enzymatic reaction is routinely assayed by following the change in optical rotation. The optical rotation of the cellobiulose-enzyme system remained constant and no reaction product or loss of keto-disaccharide was detected by paper chromatography. It is apparent from these results that the keto-disaccharide is not an intermediate in the enzymatic epimerization of cellobiose to glucosylmannose.

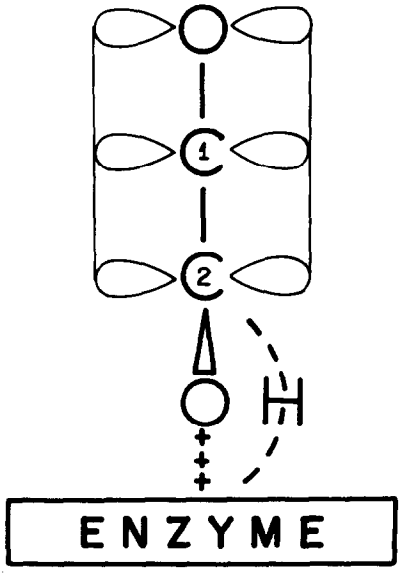

Figure 2. A proposed carbanion-type intermediate showing carbons one and two, the "leaving" hydrogen, and the stabilization of carbon-two by the enzyme. The pi bond-character should partially stabilize the near planar intermediate. The "entering" hydrogen is not shown, however, if it entered remote from the "leaving" hydrogen, the result would be an inverted-exchanged product.

On the basis of these results and the fact that we have not observed other possible intermediate compounds, we propose the following mechanism for the epimerization of the disaccharide (Fig. 2). The mechanism would require the enzymatic stabilization of the hydroxyl group at carbon-two in order to prevent keto formation. The initial step apparently is the attack by a basic group of the enzyme on the hydrogen alpha to the carbonyl leading to subsequent removal of this atom. This would result in the formation of a carbanion-type intermediate with a substantial portion of the negative charge on the carbonyl oxygen. This intermediate would have a near planar configuration at carbon-two (8). At the same time a hydrogen either from the solvent or an exchangeable site on the enzyme could become hydrogen-bonded presumably at the remote face from the leaving hydrogen. Thus the planar intermediate is hydrogen bonded at one face by the "leaving" hydrogen and at an opposite face by the "entering" hydrogen. Capture of the "entering" hydrogen by the intermediate gives an inverted-exchanged product.

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