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THE INCORPORATION OF TRITIUM FROM TRITIUM-ENRICHED WATER INTO UDP-*N*-ACETYLGLUCOSAMINE AND UDP-*N*-ACETYLMANNOSAMINE CATALYZED BY UDP-*N*-ACETYLGLUCOSAMINE 2-EPIMERASE FROM *ESCHERICHIA COLI*

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Summary

Uridine diphosphate *N*-acetylglucosamine 2-epimerase from *Escherichia coli* 014 K7 H^- catalyzes the reversible epimerization of uridine diphosphate *N*-acetylglucosamine to uridine diphosphate *N*-acetylmannosamine. During epimerization, tritium from tritium-enriched water is incorporated into both uridine diphosphate *N*-acetylglucosamine and uridine diphosphate *N*-acetylmannosamine. The position of incorporation is C-2 of the N-acetylhexosamine moieties.

Recently Kawamura et al. [1] reported the presence of an enzyme in *E. coli* 014 K7 H^- which catalyzes the reversible 2-epimerization of UDPGlcNAc to UDPManNAc. In light of this finding it has been proposed [2] that this enzyme be called UDPGlcNAc 2-epimerase and that the previously discovered UDPGlcNAc 2-epimerase from rat liver [3, 4], which catalyzes the conversion of UDPGlcNAc to UDP plus ManNAc, be called UDPGlcNAc 2-epimerase (hydrolyzing).

The mechanism of action of 2-epimerase (hydrolyzing) has been studied by several laboratories. It was first suggested by Spivak and Roseman [5] that the reaction proceeded through an epimerization step followed by hydrolysis. However, Glaser [6] subsequently reported that tritium from tritium-enriched water was incorporated at C-2 of ManNAc but was not incorporated (at detect-

Abbreviations: GlcNAc, *N*-acetyl-D-glucosamine; ManNAc, *N*-acetyl-D-mannosamine; ManNAc-ol acetate and GlcNAc-ol acetate, 1,3,4,5,6-pentaacetyl-*N*-acetylmannosaminitol and 1,3,4,5,6-pentaacetyl-*N*-acetylglucosaminitol, respectively; HexNAc, GlcNAc-ManNAc mixture; 2-epimerase; UDPGlcNAc 2-epimerase from *E. coli*; 2-epimerase (hydrolyzing), UDPGlcNAc 2-epimerase (hydrolyzing) from rat liver.

able levels) into the GlcNAc moiety of UDPGlcNAc. This observation was not consistent with the mechanism proposed by Spivak and Roseman. More recently Salo and Fletcher [7] synthesized UDPManNAc and showed it to be an alternate substrate for 2-epimerase (hydrolyzing) and suggested a mechanism involving a glycosyl enzyme and a 3-keto HexNAc intermediate summarized as follows:

$$\text{UDPGlcNAc} + \text{E} \rightarrow \text{UDP} + \text{GlcNAc-E} \rightarrow \text{3-keto-GlcNAc-E} \rightleftarrows \text{3-keto-ManNAc-E} \rightleftarrows$$

$$\text{ManNAc-E} \xrightarrow{\text{H}_2\text{O}} \text{ManNAc} + \text{E}$$

where the enzyme, or an attached coenzyme, undergoes alternate reduction and oxidation during the respective oxidation and reduction at C-3 of the HexNAc. Sommar and Ellis [8] proposed a mechanism whereby UDP is reversibly eliminated from UDPGlcNAc to give a 2-acetamidoglucal which is subsequently irreversibly hydrated to give ManNAc.

The discovery of 2-epimerase [1], which catalyzes the epimerization of the GlcNAc moiety of UDPGlcNAc to ManNAc, without concomitant hydrolytic release of ManNAc, provides an opportunity for comparative mechanistic studies, which may in time lead to an understanding of the mechanisms for both of the 2-epimerase reactions. With this thought in mind I set out to determine if 2-epimerase catalyzed the incorporation of tritium into HexNAc during the epimerization of UDPGlcNAc to UDPManNAc, and, if tritium was incorporated, to determine the position of incorporation.

UDPGlcNAc 2-epimerase was prepared from *E. coli* 014 K7 H^- as described by Kawamura et al. [1] minus the chromatography on hydroxyapatite, since my preparations of hydroxyapatite failed to bind the enzyme.

UDPGlcNAc was incubated with 2-epimerase in the presence of ^{3}HHO. The results of this experiment are shown in Table I. These results clearly show that 2-epimerase catalyzed the incorporation of tritium into UDPHexNAc. The UDPHexNAc was then hydrolyzed and the HexNAc isolated by paper chromatography (Whatman 3MM, butanol/pyridine/water, 10 : 3 : 3 v/v). The HexNAc was then reduced with sodium borohydride, passed over a small column (approx. 1 x 3 cm) of IR 120 (H^+) and the boric acid removed by repeated (3 x) evaporation to dryness with methanol. The reduced HexNAc was

TABLE I

THE INCORPORATION OF TRITIUM FROM ^{3}HHO INTO UDPGlcNAc AND UDPManNAc

UDPGlcNAc (3 μmol), 2-epimerase (0.26 units, Tris·HCl buffer pH 8.6 (4 μmol) and ^{3}HHO (to a specific activity of 2.5 mCi/mmol) in a final volume of 0.3 ml were incubated at 37° C for 30 min. The reaction was stopped by heating to 100° C for 2 min. Coagulated protein was removed by centrifugation. The supernatant was lyophilized (to recover the ^{3}HHO), the residue reconstituted in distilled water and streaked on Whatman 3MM paper. The chromatograms were irrigated with ethanol/1 M ammonium acetate, pH 7.5 (7.5 : 3, v/v). The UDPGlcNAc band was eluted and adsorbed on acid-washed charcoal (0.3 g), which was then washed with distilled water until the washings were free of radioactivity. The ultraviolet-absorbing material (UDPHexNAc) was then eluted with 50% ethanol/NH_4OH (0.2 ml of conc. NH_4OH in 100 ml of 50% ethanol) and its specific activity determined.

Reaction	Specific activity of UDPHexNAc (cpm/μmol)
UDPGlcNAc, 2-epimerase + ^{3}HHO	5000
UDPGlcNAc, 2-epimerase + ^{3}HHO (boiled control)	275

then acetylated with pyridine/acetic anhydride (1 ml of each) at 70°C for 1 h. The acetylated hexosaminitols were extracted and analyzed by gas-liquid chromatography through a 2 mm x 6-foot column of 3% ASI 50-phenyl 50-cyanopropyl on Gas Chrom Q at 250°C. This analysis indicated the presence of ManNAc-ol acetate and GlcNAc-ol acetate in a ratio of approximately 1 : 10. The ManNAc-ol acetate and GlcNAc-ol acetate peaks were collected from the gas-liquid chromatograph and counted. Both were found to be labeled with tritium.

Since it was now shown the 2-epimerase catalyzed the incorporation of tritium from ^{3}HHO into UDPGlcNAc and UDPManNAc, an assay based on this incorporation was developed. UDPGlcNAc (3 μmol) was incubated with increasing amounts of enzyme plus Tris · HCl buffer pH 8.6 (4 μmol) and ^{3}HHO (to a specific activity of 0.5 mCi/mmol) in a final volume of 0.3 ml for 20 min at 37°C. The reaction was stopped by heating at 100°C for 2 min. The supernatant was acidified to pH 1, heated at 100°C for 10 min, cooled and passed through a column of IR-120 (H^+) layered over IR 45 (OH^-) (1 ml each), followed by 2 ml of deionized water. A 0.1-ml sample of the eluant was placed in a counting vial and dried under an air stream. Beckman Fluoralloy containing 5% bbs solubalizer (10 ml) was added to the residue in the vial and it was counted. The cpm in a zero-time control were subtracted from the cpm obtained for each assay tube. A linear relationship between tritium incorporation and enzyme concentration was obtained. For the purposes of this paper, 1 unit is defined as the amount of enzyme catalyzing the incorporation of 100 cpm of tritium in 20 min under the conditions described above. All operations were conducted in a well ventilated hood.

In order to determine the position of tritium incorporation into the HexNAc moieties of UDPHexNAc, UDPGlcNAc (6 μmol), 2-epimerase (0.4 units) and ^{3}HHO (to a specific activity of 5.4 mCi/mmol) in a total volume of 0.3 ml was incubated for 30 min, followed by work-up as described in the legend to Table I. The UDPHexNAc was then hydrolyzed and the HexNAc purified by paper chromatography (butanol/pyridine/water, 10 : 3 : 3, v/v). The specific activity of the HexNAc was determined, and it was then degraded as previously described: [6, 7] first to HexN, then to arabinose, and finally to arabinonic acid. The specific activities of arabinose and arabinonic acid were determined. The results are summarized in Table II. The specific activity did not change as a result of degradation of HexNAc to arabinose, but dropped to the control value when the arabinose was oxidized to arabinonic acid. The results clearly show that the tritiated HexNAc was labeled at C-2.

TABLE II

DEGRADATION OF TRITIUM-LABELED HexNAc

Compound	Specific activity (cpm/μmol)*
HexNAc	12146
Arabinose	12602
Arabinonic acid	13**

*Zero-time boiled controls showed 361, 284 and 129 cpm/μmol, respectively for HexNAc, arabinose and arabinonic acid.

**Arabinose was diluted 8-fold before oxidation to arabinonic acid, so the comparable specific activity should be 104.

It was previously found [6] that UDPGlcNAc 2-epimerase (hydrolyzing) catalyzed the incorporation of tritium from ^{3}HHO into the C-2 position of ManNAc. I have now found that UDPGlcNAc 2-epimerase also catalyzes the incorporation of tritium from ^{3}HHO into the C-2 position of HexNAc moiety of UDPHexNAc. This incorporation is consistent with the mechanism already proposed [2], which is analogous to that of Salo and Fletcher [7] for 2-epimerase (hydrolyzing), summarized as follows:

$$E^{ox} \cdot \text{UDPGlcNAc} \rightleftarrows E^{red} \cdot \text{UDP-3-keto-GlcNAc} \rightleftarrows E^{red} \cdot \text{UDP-3-keto-ManNAc} \rightleftarrows$$

$$E^{ox} \cdot \text{UDP-ManNAc}$$

A reversible elimination of UDP could also result in formation of UDPManNAc, which corresponds to the first step of the elimination mechanism proposed [8] for 2-epimerase (hydrolyzing). However, the involvement of keto intermediates in transformations of the glycosyl moieties of the nucleoside diphosphosugars is well established (ref. 9, review) and the 3-keto mechanism is in keeping with this pattern. It should be noted that the proposed 3-keto mechanism requires the alternate reduction and oxidation of the enzyme or a coenzyme, but, to date, the enzyme has not been shown to require a coenzyme or to contain a coenzyme. Also the presence of the proposed 3-keto intermediate has not been established. Since 2-epimerase, unlike 2-epimerase (hydrolyzing) is stable, sufficient quantities of it may be purified to establish these points experimentally.

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