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URIDINE DIPHOSPHATE *N*-ACETYL-D-GLUCOSAMINE 2-EPIMERASE FROM RAT LIVER

II. STUDIES ON THE MECHANISM OF ACTION*

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SUMMARY

1. Kinetic studies of product inhibition of the reaction catalyzed by UDP-*N*-acetylglucosamine 2-epimerase were consistent with an ordered mechanism, where UDP was the first product released followed by irreversible formation of *N*-acetylmannosamine.

2. Double reciprocal plots of initial velocity versus UDP-*N*-acetylglucosamine concentration at several levels of UDP showed the inhibition produced by UDP was linear noncompetitive with $K_{i\ \text{slope}}$ less than $K_{i\ \text{intercept}}$.

3. From the evidence presented, a two step mechanism is proposed which involves the elimination of UDP from UDP-*N*-acetylglucosamine to give a 2-acetamidoglucal intermediate which is subsequently converted to *N*-acetylmannosamine.

INTRODUCTION

UDP-*N*-acetylglucosamine 2-epimerase differs markedly from both epimerases[1,2] involved in the formation of D-mannosamine derivatives in that it is specific for UDP-*N*-acetylglucosamine (UDP-GlcNAc), is inactive with the glycolyl derivative, and the reaction it catalyzes is not reversible[3]. The reaction is unique among those of nucleotide sugars since the product is not nucleotide bound. When the UDP-GlcNAc 2-epimerase reaction was carried out in tritiated water, [2-^{3}H]*N*-acetylmannosamine (ManNAc) was formed, but no tritium was found in the unreacted UDP-GlcNAc[4]. The reaction was originally thought to proceed by epimerization followed by hydrolysis[3], a reaction sequence requiring UDP-ManNAc as an intermediate. Recently, Salo and Fletcher[5,6] synthesized this compound and showed it to be an alternative substrate rather than an intermediate. When UDP-ManNAc was used as substrate in tritiated

Abbreviations: GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; 2-acetamidoglucal, 2-acetamido-1,2-dideoxy-D-*arabino*-hex-1-enopyranose.

* A preliminary report[8] of this work was presented at the 62nd annual meeting of the American Society of Biological Chemists, San Francisco, California, June 1971.

water, the [2-^{3}H]ManNAc formed had a slightly lower specific activity than that formed from UDP-GlcNAc under identical conditions[6]. These observations led to the proposal of a mechanism involving a keto intermediate and requiring a cofactor such as NAD^+, possibly bound to the enzyme[6].

In the previous paper[7] we described the purification, catalytic and regulatory properties of UDP-GlcNAc 2-epimerase from rat liver. We have extended these studies to an investigation of its mechanism of action. This paper reports such studies carried out with the purified enzyme described in the preceding paper[7]. Product inhibition studies were consistent with an ordered mechanism, where UDP was the first product released followed by irreversible formation of ManNAc[8]. A two-step mechanism is proposed for the UDP-GlcNAc 2-epimerase reaction which involves the elimination of UDP from UDP-GlcNAc to give a 2-acetamidoglucal intermediate which is subsequently converted to ManNAc. This mechanism, which does not require the presence of a cofactor, provides a rationale for the unusual specificity of this enzyme for a nucleotide sugar in ManNAc formation.

EXPERIMENTAL PROCEDURE

UDP-GlcNAc 2-epimerase was prepared and assayed as previously described[7]. Because of the extreme instability of the enzyme, investigations were carried out immediately after the completion of the purification procedure[7]. The specific activity of the enzyme was routinely about 0.25 I.U. per mg protein (1 unit = 1 μmole ManNAc produced per min).

Materials

N-Acetylglucosamine was purchased from Pfanstiehl Laboratories, Inc. 2-Acetamido-1,2-dideoxy-D-*arabino*-hex-1-enopyranose (2-acetamidoglucal) was synthesized as described by Salo and Fletcher[9] for the preparation of 2-acetamidoglycals. The acetylated oxazoline derived from *N*-acetylglucosamine was prepared according to the procedure of Pravdic *et al.*[10]. This compound readily isomerized when heated at 100 °C in tetramethylurea containing a trace of *p*-toluenesulfonic acid. De-o-acetylation with sodium methoxide gave 2-acetamidoglucal in crystalline form[9]. Alumina and silica-gel thin-layer chromatography plates were obtained from Analtech. All other chemicals were the same as in the previous communication[7].

Thin-layer chromatography and paper electrophoresis

2-Acetamidoglucal was separated from *N*-acetylhexosamines on silica-gel plates using a 5% acetic acid in acetone solvent system. High voltage paper electrophoresis was carried out on Schleicher and Schuell 2043B paper in a flat plate high voltage electrophoresis cell (Camag) at a potential gradient of 75 V/cm for 30 min. The buffer used for the separation of ManNAc and GlcNAc[11] was 0.05 M sodium tetraborate (pH 9.0). The sample was applied as a thin zone on the paper. Compounds were visualized by yellow fluorescence after spraying guide strips with 0.5 M alcoholic NaOH and heating at 100 °C for 5 min[11]. ManNAc was eluted from the remainder of the paper and assayed by a modification of the Morgan–Elson procedure[12].

Product inhibition

Product inhibition studies were performed in the presence of given amounts of UDP using both the colorimetric and radioactive assays for measurement of reaction velocity[7]. Kinetic parameters were determined from a computer analysis of the data using the method of Wilkinson[13]. $K_{i\ \text{slope}}$ and $K_{i\ \text{intercept}}$, as described by Cleland[14], were determined from a regression analysis of the slopes and intercepts plotted against UDP concentrations.

RESULTS AND DISCUSSION

Experiments were performed under conditions where velocity was linear with respect to time and enzyme concentration.

Order of release of products and inhibition by UDP

Product inhibition studies have been used to determine the order of substrate

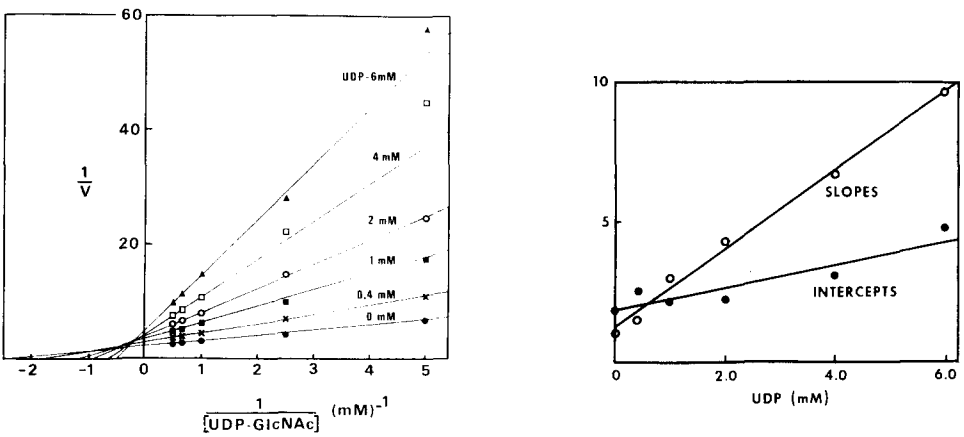

Fig. 1. Effect of UDP on reaction velocity. Lineweaver–Burk plots for UDP-GlcNAc 2-epimerase in the presence of different levels of UDP. Each reaction vessel contained: 200 mM Tris–HCl (pH 7.5); 50 mM $MgSO_4$; 0.02 unit of enzyme, UDP-GlcNAc and UDP as indicated in a final volume of 1 ml. Incubations were conducted at 37 °C for 20 min and ManNAc was assayed by the colorimetric procedure[7]. Velocity is expressed as μmoles ManNAc formed in 20 min. Slopes and intercepts were determined from a computer-generated linear regression analysis of the data. Correlation coefficients for the lines were in all cases greater than 0.975.

Fig. 2. Replots of the slopes and intercepts on the ordinate from Fig. 1 as a function of the UDP concentration. Correlation coefficients for the lines were greater than 0.992.

addition and product release in multisubstrate or multiproduct reactions[14]. When the epimerase reaction was run in the presence of each product individually, it was found that concentrations of ManNAc up to 20 mM had no effect on the reaction. UDP, however, proved to be a noncompetitive inhibitor. Reciprocal plots of velocity *versus* UDP-GlcNAc concentration at several levels of UDP are shown in Fig. 1. This figure clearly demonstrates the noncompetitive nature of the inhibition produced by UDP. The values of K_i are readily obtained by replotting the slopes and intercepts of Fig. 1

against UDP concentration as shown in Fig. 2. The linear plots gave values of 0.9 mM and 4.8 mM, respectively, for $K_{i\ \text{slope}}$ and $K_{i\ \text{intercept}}$.

These findings are consistent with the kinetic mechanism:

$$E + S \overset{1}{\rightleftharpoons} ES \overset{2}{\rightleftharpoons} EQ + P$$

$$EQ \overset{3}{\rightarrow} E + Q$$

in which the formation of UDP is reversible while the formation of ManNAc is irreversible, as has been shown by Glaser[4] and also Salo and Fletcher[6]. When steady state kinetics are applied:

$$v = \frac{k_1 k_2 k_3 S E_0}{(k_1 + k_2)k_3 + k_{-1}k_{-2}P + k_1(k_2 + k_3)S + k_1 k_{-2} S P} \tag{1}$$

Define:

$$V = \frac{k_2 k_3 E_0}{k_2 + k_3}$$

$$K_m = \frac{(k_{-1} + k_2)k_3}{k_1 (k_2 + k_3)}$$

$$K_p = \frac{(k_{-1} + k_2)k_3}{k_{-1} k_{-2}}$$

$$K_{ip} = \frac{k_2 + k_3}{k_{-2}}$$

where K_{ip} is the inhibition constant for P and K_p is the Michaelis constant for P in the reverse direction.

Then:

$$v = \frac{VS}{K_m\left(1 + \frac{P}{K_p}\right) + S\left(1 + \frac{P}{K_{ip}}\right)} \tag{2}$$

In double reciprocal form:

$$\frac{1}{v} = \frac{K_m}{V}\left(1 + \frac{P}{K_p}\right)\frac{1}{S} + \frac{1}{V}\left(1 + \frac{P}{K_{ip}}\right) \tag{3}$$

Compare Eqn 3 with Cleland's[14] equation for linear noncompetitive inhibition where:

$$\frac{1}{v} = \frac{K_a}{V}\left(1 + \frac{I}{K_{i\ \text{slope}}}\right)\frac{1}{A} + \frac{1}{V}\left(1 + \frac{I}{K_{i\ \text{intercept}}}\right)$$

The derivation of the rate equation for the epimerase reaction gives linear noncompetitive inhibition for P in the same manner as described by Cleland[14], with the first product released, UDP, a noncompetitive inhibitor of the reaction. The product inhibition studies (Figs 1 and 2) are consistent with an ordered mechanism showing the reversible release of UDP prior to the irreversible formation of ManNAc.

2-Acetamidoglucal as an intermediate

The mechanism proposed by Salo and Fletcher[6] for the 2-epimerase reaction takes into account that UDP-ManNAc, rather than serving as an intermediate in the

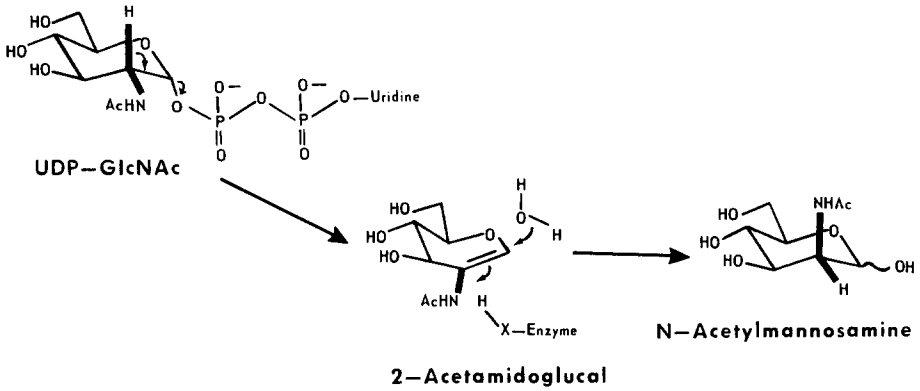

Fig. 3. Proposed mechanism of action of UDP-GlcNAc 2-epimerase.

transformation of UDP-GlcNAc to ManNAc as previously suggested[3], may simply be an alternate substrate for the enzyme. The mechanism involves the formation of glycosyl enzyme complexes and keto intermediates and requires the 2-epimerase to contain or utilize a cofactor such as NAD^+ (or $NADP^+$). Since a requirement for NAD^+ has not been shown, we suggest an alternative mechanism for the formation of ManNAc by the 2-epimerase. This mechanism shown in Fig. 3 is consistent with published data and the results of the product inhibition studies (Fig. 1).

The first product released, UDP, is eliminated by a favorable *trans*-elimination from UDP-GlcNAc to give 2-acetamidoglucal (Fig. 3). In the presence of tritiated water a replaceable proton on the enzyme would then be donated stereospecifically to the double-bonded acetamidoglucal intermediate to give the labeled product, [2-^{3}H]-ManNAc. The irreversible formation of ManNAc from the 2-acetamidoglucal explains the observed absence of tritium incorporation into substrate[4,6]. The unfavorable *cis*-elimination of UDP from UDP-ManNAc to give 2-acetamidoglucal could be favored by a possible cyclic transition state involving the phosphate oxygen and the proton at C-2. Formation of a 2-acetamidoglucal intermediate from both UDP-GlcNAc and UDP-ManNAc would explain the similarity of labeling in the presence of tritiated water as well as the specificity of the label position observed by Salo and Fletcher[6]. Thus, simple elimination of UDP is followed by the addition of water to effect epimerization. This mechanism also provides an explanation for the unique requirement of the 2-epimerase for a nucleotide sugar to form ManNAc, which is not nucleotide bound.

In order to test the proposed reaction mechanism we synthesized 2-acetamidoglucal using the method of Pravdic *et al.*[10] for acetamidoglycal derivatives. Two technical difficulties prevented quantitation of results when the acetamidoglucal was used as a substrate for the 2-epimerase. The acetamidoglucal gave a positive reaction in the Morgan–Elson colorimetric procedures[7] for the determination of ManNAc and in solution was rapidly converted to GlcNAc. However, when the acetamidoglucal was incubated with UDP-GlcNAc 2-epimerase a compound with chromatographic and electrophoretic properties similar to ManNAc was formed. This compound was separable from the acetamidoglucal on silica-gel plates in an acetone–acetic acid solvent system. On high voltage paper electrophoresis[11] the compound migrated to the same position

as ManNAc. This compound was not formed when the acetamidoglucal was incubated with buffer alone. The above results suggest the reaction mechanism shown in Fig. 3 is plausible and consistent with published data.

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