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

I. CATALYTIC AND REGULATORY PROPERTIES

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SUMMARY

1. UDP-*N*-acetylglucosamine 2-epimerase, the enzyme catalyzing the formation of UDP and *N*-acetylmannosamine from UDP-*N*-acetylglucosamine, was purified 100–200-fold from rat liver. The final enzyme preparation was extremely unstable with activity completely disappearing within a few hours.

2. The epimerase reaction had double pH optima of 7.1 and 7.9 and a K_m of 0.2 mM for UDP-*N*-acetylglucosamine was found.

3. The allosteric inhibition of the enzyme by CMP-*N*-acetylneuraminic acid was investigated in detail. A sigmoidal inhibition curve was obtained suggesting cooperative homotropic effects. A Hill plot yielded an interaction coefficient of $\bar{n} = 4$.

4. The regulatory significance of the epimerase reaction to nucleotide sugar metabolism is discussed.

INTRODUCTION

UDP-*N*-acetylglucosamine (UDP-GlcNAc) is a key intermediate in the biosynthesis of the oligosaccharide side chains of glycoproteins and mucopolysaccharides. In addition to being the donor of GlcNAc residues of these macromolecules, the compound is situated at a branch point for the synthesis of other nucleotide sugars[1]. The initial enzyme responsible for the biosynthesis of CMP-*N*-acetylneuraminic acid (CMP-NANA) is UDP-GlcNAc 2-epimerase. This enzyme which catalyzes the formation of UDP and *N*-acetylmannosamine from UDP-GlcNAc was first described by Cardini and Leloir[2] and Comb and Roseman[3] and further purified and studied by Spivak and Roseman[4]. Feedback inhibition of a crude enzyme preparation by CMP-NANA has been reported by Kornfeld *et al.*[5]. During the course of their studies Spivak and Roseman[4] reported an apparent lag in the formation of UDP which led them to suggest that the enzyme reaction proceeds by two steps, epimerization followed by hydrolysis, a reaction sequence requiring UDP-*N*-acetylmannosamine as an inter-

Abbreviations: GlcNAc, *N*-acetylglucosamine; ManNAc, *N*-acetylmannosamine; NANA, *N*-acetylneuraminic acid; PEP, phosphoenolpyruvate.

mediate. Recently, Salo and Fletcher[6] synthesized this compound and showed it to be an alternative substrate rather than an intermediate[7]. UDP was also reported to stabilize the enzyme[4]. We have recently presented evidence for noncompetitive product inhibition by UDP[8,9] and have also observed significantly lower K_m values for the enzyme[8] than Spivak and Roseman[4]. The present study was undertaken to obtain information concerning these apparent differences. This paper describes the purification of UDP-GlcNAc 2-epimerase from rat liver, its kinetic and regulatory properties and its relationship to other enzymes involved in the biosynthesis of sugar nucleotides. Part of this work has appeared in preliminary form[8].

MATERIALS AND METHODS

Chemicals

UDP-GlcNAc and phosphoenolpyruvate (PEP) were purchased from Boehringer Mannheim; NADH, lactate dehydrogenase (L-lactate:NAD^+ oxidoreductase, EC 1.1.1.27), Type II, pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40), Type II; dithiothreitol and *N*-acetylmannosamine (ManNAc) were from Sigma Chemical Co. UDP and uridine were obtained from P–L Biochemicals, while UDP-[1-^{14}C]GlcNAc was purchased from New England Nuclear. DEAE-cellulose (Type 20) was obtained from Schleicher and Scheull and precycled as described[4]. *N*-Acetylneuraminic acid (NANA) was obtained from Pfanstiehl Laboratories, Inc. Enzyme grade $(NH_4)_2SO_4$ and polymyxin sulfate were purchased from Mann, and calcium phosphate gel from Nutritional Biochemicals. CMP-*N*-acetylneuraminic acid (CMP-NANA) was prepared enzymatically as described by Kean and Roseman[10] except for the following modification in the preparation of CMP-*N*-acetylneuraminic acid synthetase. The precipitate from a 45–65% satd $(NH_4)_2SO_4$ fractionation of a crude supernatant extract of steer submaxillary glands was used as the source of the synthetase enzyme, eliminating the lengthy DEAE-cellulose column purification steps. All other chemicals were of the highest quality available from commercial sources.

Analytical techniques

Protein was determined by the method of Lowry *et al.*[11] using crystalline bovine serum albumin as a standard. *N*-Acetylmannosamine was measured by a modification[12] of the Morgan–Elson procedure as adapted by Spivak and Roseman[4]. CMP-NANA was measured by the thiobarbituric acid assay of Warren[13] after reduction with sodium borohydride.

Enzyme assays

Three methods of assaying UDP-GlcNAc 2-epimerase were used. In each case enzymatic activities were calculated from initial rates measured under conditions of linearity with respect to time and protein concentration.

Assay a. This procedure, based on the measurement of ManNAc by means of a modified Morgan–Elson procedure[4], was used for routine studies and in the purification of the enzyme. The presence of dithiothreitol in enzyme fractions had no apparent effect on the colorimetric reaction. The standard incubation mixture was as follows: 0.5 μmole of UDP-GlcNAc; 50 μmoles of Tris–HCl buffer (pH 7.5); 12.5 μmoles $MgSO_4$ and enzyme in a total volume of 0.25 ml. Incubation was carried out

at 37 °C for 20 min and the reaction was stopped by boiling for 2 min. After centrifugation, aliquots of the supernatant were used for the colorimetric determination of ManNAc[4]. Controls consisted of incubation mixtures lacking enzyme, or containing heat-inactivated enzyme.

Assay b. A coupled enzyme spectrophotometric assay was used in which UDP-GlcNAc 2-epimerase was coupled with pyruvate kinase and lactate dehydrogenase in the presence of phosphoenolpyruvate (PEP) and NADH. Concentrations of auxiliary enzymes were chosen to provide optimum conditions for the assay as defined by McClure[14]. Routine assays were carried out in final volumes of 0.25 ml using a Gilford recording spectrophotometer with a 37 °C constant temperature attachment. Routine assay mixtures were prepared so that the final concentrations after the addition of enzyme were 200 mM Tris–HCl buffer (pH 7.5); 50 mM $MgSO_4$; 2 mM UDP-GlcNAc; 2 mM sodium PEP; 0.038 mM NADH; 10 I.U. of pyruvate kinase; 15 I.U. of lactate dehydrogenase and 3–5 munits of enzyme. Particular care was taken to use PEP with minimal amounts of pyruvate. Rates were corrected for controls run in the absence of substrate.

Assay c. This was a radioactive assay in which the standard incubation mixture contained 200 mM Tris–HCl buffer (pH 7.5); 50 mM $MgSO_4$; 2 mM UDP-[1-^{14}C]-GlcNAc (0.125 μCi per μmole) and the enzyme to be assayed in a final volume of 0.1 ml. After incubation for 20 min at 37 °C, the reaction was stopped by heating at 100 °C and the denatured protein removed by centrifugation. A 0.06-ml aliquot was removed from the supernatant and placed on a 3-ml column of Dowex 1-X2 formate resin. The column was washed with 0.9 ml of water, and the labeled ManNAc was eluted with an additional 2 ml of water. Radioactivity was estimated with a Packard Tri-Carb liquid scintillation counter using Bray's solution[15]. Controls consisted of incubation mixtures containing heat-inactivated enzyme.

Unit

One unit of UDP-GlcNAc 2-epimerase is defined as the amount of enzyme that will catalyze the formation of 1 μmole of ManNAc per min at 37 °C.

Estimation of kinetic parameters

K_m and V were determined from a computer generated linear regression analysis of the experimental data by the method of Wilkinson[16].

RESULTS

Purification of UDP-GlcNAc 2-epimerase

The epimerase was prepared essentially by the method described by Spivak and Roseman[4] with several minor modifications. Unless otherwise stated, all procedures were carried out at temperatures between 0 and 4 °C and all buffers used in the fractionation procedure contained 1 mM EDTA and 0.5 mM uridine. Sprague–Dawley rats weighing 150–250 g were decapitated and their livers were quickly removed and chilled on ice. The livers were weighed and homogenized with 2 vol. of ice-cold 5 mM potassium phosphate buffer (pH 7.5) in a Waring blender for 1 min. The homogenate was centrifuged for 20 min at 30 000 × g in a Sorvall refrigerated centrifuge. To 40 ml of the supernatant (crude extract) were added 15 ml of a 1%

polymyxin sulphate solution. After 5 min of stirring, the mixture was centrifuged at 30 000 × *g* for 15 min and the precipitate discarded. To the 50 ml polymyxin supernatant, 30 ml of satd $(NH_4)_2SO_4$ solution (pH 7.0) were slowly added. This was stirred for 20 min and then centrifuged at 30 000 × *g* for 10 min. This precipitate was then taken up in 30 ml of the phosphate buffer. The calcium phosphate gel and second $(NH_4)_2SO_4$ precipitate steps followed published procedures[4]. The $(NH_4)_2SO_4$ precipitate was then taken up in 5 ml of 5 mM phosphate buffer containing 0.1 mM dithiothreitol (pH 7.5) and dialyzed for 2 h against the same buffer. Following dialysis the enzyme solution was placed on a 1 cm × 7 cm DEAE-cellulose column previously equilibrated with the 5 mM potassium phosphate buffer. Elution of epimerase was as described[4]. The final 25-ml eluate was collected and 25 ml of satd $(NH_4)_2SO_4$ solution added. After 20 min the mixture was centrifuged for 10 min at 20 000 × *g*, and the precipitate redissolved in 2 ml of the potassium phosphate buffer.

The enzyme was purified 100–200-fold over the initial homogenate with an

TABLE I

PURIFICATION OF UDP-GlcNAc 2-EPIMERASE

Enzyme activity was measured by Assay a. Enzyme units are calculated as μmoles of *N*-acetylmannosamine formed per min under the standard assay conditions.

Fractions	*Total volume (ml)*	*Protein (mg/ml)*	*Total activity (units)*	*Specific activity (units/mg protein)*	*Recovery (%)*	*Purification factor*
Crude extract	40	48.3	6.5	0.002	100	1
Polymyxin supernatant	50	24.3	8.5	0.007	130	3.5
0–40% $(NH_4)_2SO_4$	30	5.4	7.4	0.045	113	22
Calcium phosphate gel	5	5.3	4.0	0.15	61	75
DEAE-cellulose eluate*	1.5	2.5	1.1	0.29	17	145

* 0–50% saturated $(NH_4)_2SO_4$ precipitate of DEAE-cellulose eluate, dissolved in phosphate buffer.

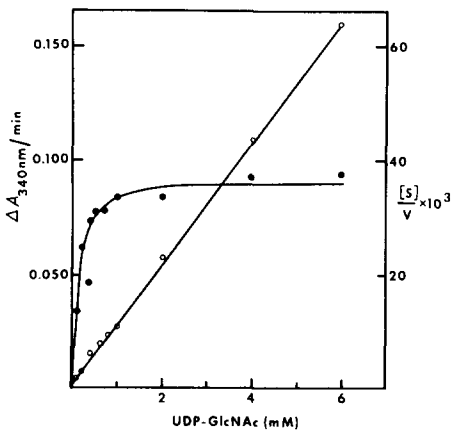
overall yield of approx. 20%. Typical data of a purification are shown in Table I. The final enzyme preparation was extremely unstable, with activity completely disappearing within a few hours. Whenever possible the purification of the enzyme and experimental investigations were carried out on the same day. The purified enzyme could, however, be stored overnight at −10 °C in a satd $(NH_4)_2SO_4$ solution with approx. 20% loss in activity. Immediately prior to use the enzyme solution was desalted by passing through a 0.7 cm × 5 cm Sephadex column previously equilibrated with the phosphate buffer.

Properties of UDP-GlcNAc 2-epimerase

Cell fractionation studies indicated enzyme activity was localized in the cell supernatant. The purified enzyme was extremely unstable, even in the presence of uridine and dithiothreitol. This lability of the enzyme prevented the determination of a meaningful molecular weight by gel filtration or sucrose gradient centrifugation methods. Attempts with crude enzyme preparations in the presence of stabilizing agents indicate a molecular weight in the approximate range of 400 000–600 000.

Kinetic studies

Enzyme kinetic experiments were performed under conditions where velocity was linear with both time and concentration of the epimerase and in the absence of enzyme-stabilizing reagents. The concentration of UDP-GlcNAc was varied using 4–12 munits of purified enzyme per ml of reaction mixture, allowing no more than 12% of the substrate to be converted to products. The effect of substrate concentration on rate of reaction using the coupled enzyme assay is shown in Fig. 1. The apparent K_m value calculated from a Woolf plot[17] using the method of Wilkinson[16] was 0.19 mM (Fig. 1). Determinations with different batches of enzyme using all three assay systems gave K_m values in the range of 0.2–0.4 mM for UDP-GlcNAc. This is an order of magnitude



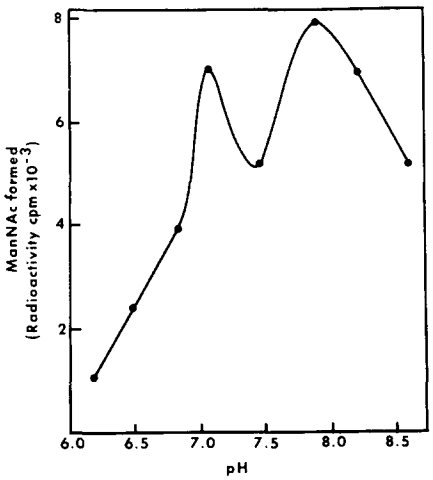


Fig. 1. Effect of UDP-GlcNAc concentration on reaction rate. Conditions of the assay and composition of the incubation mixtures were as described in Assay b, except for the varying substrate concentration. K_m was determined from a computer-generated linear regression analysis of the data. Correlation coefficient for the line was 0.992.

Fig. 2. UDP-GlcNAc 2-epimerase activity as a function of pH. Composition of the incubation mixtures and assay conditions were the same as described in Assay c, except that the volume was increased to 0.25 ml and each vessel contained 50 μmoles of Tris–maleate buffer at the indicated pH. Incubations were for 20 min at 37 °C.

less than the value of 2 mM reported by Spivak and Roseman[4]. When this phenomena was further investigated, we found apparent K_m values for the enzyme to be larger when there is extensive conversion to products. As a corollary to the determination of kinetic constants, data were also plotted according to the empirical Hill equation[18]. Within the range of substrate concentration covered, the value of $\bar{n}$ in the Hill plot was unity signifying that no cooperative homotropic effects occur with UDP-GlcNAc.

Effect of pH on enzyme activity

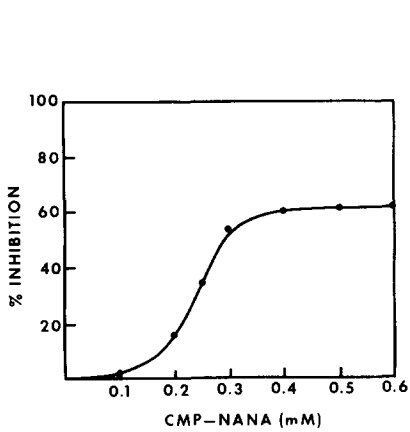
The pH dependence of the epimerase was studied using a series of Tris–maleate buffers covering the pH range 6.2–8.5. The pH values were determined using a microelectrode. The double pH optima already reported[4] were found with maximum activity at pH 7.1 and 7.9 (Fig. 2).

Stoichiometry

A combination of Assay a and Assay b was employed to determine the ratio of UDP to ManNAc. UDP was continuously measured by the coupled enzyme assay while ManNAc and UDP-GlcNAc were determined colorimetrically at the completion of a 20-min incubation period. Estimated in this manner, approximately equivalent amounts of UDP and ManNAc were formed while an equimolar amount of UDP-GlcNAc was consumed.

Inhibition by CMP-NANA

A crude preparation of the epimerase was shown by Kornfeld *et al.*[5] to be subject to feedback inhibition by CMP-NANA. The sigmoidal nature of the inhibition curve suggested the possibility of cooperative homotropic effects[19] in the presence of CMP-NANA. These experiments were repeated and confirmed using a purified preparation of the enzyme. The affinity of the purified epimerase for CMP-NANA increased as it became more saturated with the inhibitor; resulting in a sigmoidal shape curve when



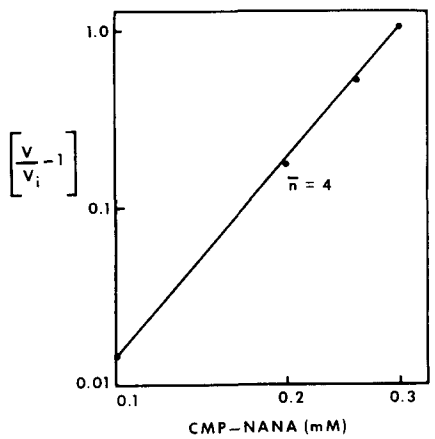


Fig. 3. Effect of CMP-NANA concentration on reaction rate. The enzyme was assayed under the standard conditions described in Assay c, except that CMP-NANA was added at the concentrations indicated.

Fig. 4. Hill plot for CMP-NANA inhibition of the epimerase plotted according to the equation: $\log (v/v_i - 1) = \bar{n} \log I + \log 1/K_i$ where v is enzyme activity in the absence of inhibitor and v_i is activity in the presence of a given concentration of CMP-NANA.

CMP-NANA concentration was plotted against percent inhibition as shown in Fig. 3. Maximum inhibition, however, was only of the order of 60%. Since the enzyme appears to be allosteric in nature, the saturation curve for the enzyme–CMP-NANA complex may be dependent on the treatment of the enzyme prior to the experiment. Desensitization of the epimerase to the inhibition produced by CMP-NANA was apparent with several preparations of the enzyme.

The sigmoidal nature of the inhibition curve is indicative of interactions between the CMP-NANA binding sites. Levitzki and Koshland[20] have shown with an enzyme subject to the allosteric effects of an inhibitor, the Hill equation[18] may be transformed to:

$$\log\left(\frac{v}{v_i} - 1\right) = \bar{n} \log I + \log \frac{1}{K_i}$$

where v is the velocity of reaction in the absence of inhibitor, and v_i is velocity in the presence of inhibitor. As shown in Fig. 4 a plot of $\log (v/v_i) - 1)$ *vs* $\log$ [CMP-NANA] gave a straight line with a slope of 4 suggesting the presence of multiple binding sites for CMP-NANA on the epimerase.

DISCUSSION

A modification of the purification procedure described by Spivak and Roseman[4] for UDP-GlcNAc 2-epimerase from rat liver permits isolation of the enzyme with a yield of about 20%. While the purified form of the enzyme is extremely unstable, several kinetic and regulatory properties were studied in detail. The K_m value reported here for UDP-GlcNAc (0.2–0.4 mM) is substantially less than the value of 2 mM quoted by Spivak and Roseman[4]. We have found apparent K_m values for this enzyme to be larger when there is extensive conversion to products, and, in addition, have observed noncompetitive inhibition by UDP[8,9]. Consequently, we believe the lower values to be a more accurate reflection of the true K_m for the enzyme. The lag period reported with several epimerase preparations[4] using the coupled enzyme assay can be explained by the use of suboptimal levels of auxiliary enzymes[14]. With the assay system used in the present experiments, which employs more optimal levels of auxiliary enzymes, we have never observed a lag period prior to the linear oxidation of NADH. The significance of the double pH optima of 7.1 and 7.9, while confirming the observations of Spivak and Roseman[4], is not presently apparent. The extreme lability of the enzyme has prevented further purification to see if there are possibly two forms of the enzyme.

The studies reported here show UDP-GlcNAc 2-epimerase exhibits properties characteristic of enzymes subject to feedback inhibition. The inhibition curve obtained with increasing CMP-NANA concentration was sigmoidal as reported for the crude enzyme by Kornfeld *et al.*[5]. The observation of cooperative homotropic effects when the Hill equation was applied to the kinetics of inhibition by CMP-NANA suggests the presence of multiple binding sites for the ligand on the epimerase. Selective desensitization to feedback inhibition while maintaining catalytic activity has been cited as evidence for the existence of separate regulatory and catalytic sites on enzyme molecules. Such studies have not been successful with the epimerase because of the general lability of the enzyme. Further investigations, with a more purified and stable epimerase preparation, will be required to resolve the nature of the interactions between CMP-NANA and the enzyme.

UDP-GlcNAc and CMP-NANA are precursors for the biosynthesis of glycoproteins and mucopolysaccharides. The synthesis of CMP-NANA involves a series of reactions starting with ManNAc[1,5]. Two different enzymes have been shown to carry out the synthesis of ManNAc in mammalian tissues, *N*-acylglucosamine 2-epimerase[21,22] and the enzyme described in the present report, UDP-GlcNAc 2-epimerase. *N*-Acylglucosamine 2-epimerase is readily reversible with the equilibrium favoring *N*-acylglucosamine[21] while the reaction catalyzed by UDP-GlcNAc 2-epimerase is not detectably reversible[4]. This irreversible reaction is thus biologically

important for the synthesis of CMP-NANA and macromolecules containing sialic acid. The observation that the enzyme is markedly inhibited by CMP-NANA may be another example of feedback inhibition of a metabolic pathway which is important physiologically.

UDP-GlcNAc occupies a central position in the pathways of aminosugar metabolism and in the biosynthesis of glycoproteins and mucopolysaccharides. From a regulatory viewpoint the compound is situated at a key metabolic junction. Two separate biosynthetic pathways are known to lead to the synthesis of UDP-GlcNAc. *De novo* synthesis of the aminosugar component involves the enzyme L-glutatamine:D-fructose-6-phosphate aminotransferase[5,23] (EC 2.6.1.16) which catalyzes the transamination of fructose 6-phosphate with glutamine. The alternative pathway utilizes GlcNAc with ATP:*N*-acetyl-D-glucosamine-6-phosphotransferase[24,25] (GlcNAc-kinase) as the first enzyme in a salvage pathway. Both the aminotransferase[5,23] and GlcNAc-kinase[26] are strongly inhibited by UDP-GlcNAc. Modulation of the activities of both these enzymes is essential since any accumulation of UDP-GlcNAc will automatically prevent its own synthesis. The intracellular concentration of UDP-GlcNAc in the liver is approx. 0.4 mM, calculated from the data of Bates and Handschumacher[27]. Since this concentration is far greater than the K_i of the aminotransferase[5,23] and of the kinase[26] for this ligand, and assuming no intracellular compartmentation, the normal activity of the enzymes *in vivo* should be that displayed by heavily inhibited enzymes. Release of the inhibition will allow for the rapid synthesis of nucleotide sugar precursors without *de novo* synthesis of enzymes. The irreversible reaction catalyzed by UDP-GlcNAc 2-epimerase provides a mechanism for removal of UDP-GlcNAc and hence release of inhibition. The flow of UDP-GlcNAc would proceed along the biosynthetic pathway to CMP-NANA. Thus when a change in the rate of glycoprotein and mucopolysaccharide synthesis occurs, the cell would be able to rapidly alter its rate of synthesis of both UDP-GlcNAc and CMP-NANA. The regulatory significance of UDP-GlcNAc 2-epimerase suggests that this little studied enzyme may play an important role in nucleotide sugar metabolism.

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