STUDIES ON THE MECHANISM OF ACTION OF UDP-D-GLUCOSE DEHYDROGENASE FROM BEEF LIVER*

J. G. SCHILLER, A. M. BOWSER, AND DAVID SIDNEY FEINGOLD†
Department of Microbiology, School of Medicine, University of Pittsburgh, Pittsburgh, Pennsylvania 15213 (U. S. A.)
(Received May 19th, 1971; accepted in revised form July 20th, 1971)

ABSTRACT

As a first step towards determining the mechanism of action of UDP-Glc dehydrogenase, the fate of the C-6 hydrogen atoms of UDP-Glc was studied. The data presented indicate that, in the NAD-linked conversion of UDP-Glc to UDP-GlcA, both C-6 hydrogen atoms of UDP-Glc are directly transferred to NAD.

INTRODUCTION

D-Glucuronic acid (GlcA), the most abundant uronic acid, is widely distributed in nature, occurring in plant and bacterial polysaccharides and in D-glucosiduronic acids and polysaccharides of animals.

In 1951 Dutton and Storey[1] showed that, in rabbit liver, uridine 5′-(α-D-glucopyranosyluronic acid pyrophosphate) (UDP-GlcA) acted as glycosyl donor in synthesis of D-glucosiduronic acids. Strominger *et al.*[2] were the first to demonstrate formation of UDP-GlcA by the NAD-linked dehydrogenation of UDP-Glc catalyzed by UDP-Glc dehydrogenase (UDP-Glc:NAD oxidoreductase, EC 1.1.1.22). The enzyme has subsequently been demonstrated in a large variety of animals, higher plants[3], fungi[4], and bacteria[5,6].

UDP-Glc dehydrogenase is unusual in that it catalyzes a 4-electron transfer; in other words, 2 moles of NAD are reduced per mole of UDP-Glc oxidized. The enzyme has been shown to have B specificity in respect to NAD reduction[7]; however, the ultimate source of the hydrogen atoms transferred to NAD during the reaction has not been investigated.

In this paper we present a study of the fate of the C-6 hydrogen atoms of UDP-Glc when it is oxidized by UDP-Glc dehydrogenase.

MATERIALS AND METHODS

Materials

UDP-Glc, UDP-GlcA, NAD, nicotinamide, and lactic acid were purchased

*Supported by a Grant (GM-08820) from the United States Public Health Service.
†Research Career Development Award (1-K3-GM-28,296) grantee of the National Institutes of Health, United States Public Health Service.

from Sigma Chemical Co., St. Louis, Mo. Glc-*6-t*, and Omnifluor were from New England Nuclear Corp., Boston, Mass. Lactic acid dehydrogenase (EC 1.1.1.27) was obtained from the Worthington Biochemical Corp., Freehold, N. J. All other chemicals were of reagent grade.

Methods

I. Synthesis of UDP-Glc-6-t. — UDP-Glc-*6-t* was prepared from Glc-*6-t* and UTP essentially as described by Wright and Robbins[8], by utilizing a crude extract from bakers' yeast that contained UDP-Glc pyrophosphorylase (EC 2.7.7.9). Crude UDP-Glc-*6-t*, which was separated from the reaction mixture by chromatography for 17 h in solvent II, was desalted by chromatography in solvent I for 48 h, and then recovered from the origin of the chromatogram and subjected to paper electrophoresis at 1600 V and 34–45 mA in ammonium formate buffer as described. The compound having the mobility of UDP-Glc was eluted and its specific radioactivity determined by a combination of enzymic assay and liquid scintillation spectrometry as follows: A sample of UDP-Glc that contained a known amount of radioactivity was completely oxidized with homogeneous UDP-Glc dehydrogenase and excess NAD in a quartz cuvette under the conditions described for the standard enzyme-assay[9]. The specific activity of UDP-Glc-*6-t* determined in this way was 31.6 μCi/μmole. The discrepancy between this value and 27.6 μCi/μmole, the specific activity of the Glc-*6-t*, indicated that not all the material isolated as UDP-Glc was converted into UDP-GlcA by the enzyme. The presence of approximately 12% of an (unknown) impurity in the isolated UDP-Glc was confirmed by chromatography in solvent I of an acid hydrolyzate (0.1M HCl for 30 min at 100°) of the preparation, demonstrating a radioactive component of different mobility from that of D-glucose.

*II. Incubation of UDP-Glc-*6-t *with UDP-Glc dehydrogenase.* — The reaction was performed in a quartz cuvette at 30° so that its progress could be monitored in the spectrophotometer; otherwise the protocol was as described by Krakow *et al.*[7] with a total volume of 1.0 ml for both the reaction mixture and the control. An aliquot of a 70% alcoholic solution containing the UDP-Glc-*6-t* was first added to each cuvette and the solvent removed by evaporation in a stream of nitrogen. The final concentration of substances in each cuvette was: 0.1M glycylglycine buffer (pH 8), 1 mM NAD, 2.75 μM UDP-Glc-*6-t* (27.6 μCi/μmole). Homogeneous UDP-Glc dehydrogenase obtained from beef liver[9] (2 μl, specific activity 3.2) was then added to the reaction cuvette. When the reaction was complete, 0.02 ml of sodium pyruvate (18 mM) and 0.02 ml of lactic acid dehydrogenase (EC 1.1.1.27) (2 mg/ml in 0.01 M NaCl) was added to each cuvette. When all the NADH present in the reaction mixture had been oxidized, 0.05-ml aliquots were removed from each vessel, diluted with 0.45 ml of water, lyophilized, and the recovered water assayed for radioactivity.

In addition, aliquots (0.45 ml) were removed from each cuvette and placed in separate vessels containing 7 ml of 3.6M lactic acid. After the addition of 2–3 drops of concentrated sulfuric acid, these solutions were continuously extracted with diethyl ether for 65 h. The ether was evaporated from the extract and the residual lactic acid

was converted to its *p*-phenylphenacyl ester[10]. The ester was then recrystallized from acetone to constant m.p. (150–151°).

The remaining portions of the reaction and control mixtures were diluted to 7.5 ml with 0.215M sodium phosphate buffer[11], p.H 9.6, and hydrolyzed by heating for 11 min at 100° in order to liberate nicotinamide. Completeness of hydrolysis was confirmed by assaying an aliquot of each hydrolyzate for intact NAD with UDP-Glc dehydrogenase (EC 1.1.1.22) in the presence of excess UDP-Glc. Carrier nicotinamide (300 mg) was then added to the remainder of each hydrolyzate and the solution was titrated to pH 7 with 0.5M H_2SO_4. The hydrolyzates were continuously extracted with ether for 22 h and the recovered nicotinamide was recrystallized from benzene to a constant m.p. (128–129°).

III. Paper chromatography and electrophoresis. — All descending paper chromatograms and electropherograms were run on Whatman 3 MM paper. Paper strips were scanned for radioactivity in a Tracerlab 4 pi scanner (Tracerlab, Waltham, Mass.). Paper electrophoresis was performed in a Savant flat-plate electrophoresis instrument (Savant Instruments, Inc., Hicksville, N. Y.). Radioactive materials were recovered from chromatograms by elution with water in a humidified chamber.

The chromatographic solvents used were: I, *n*-propanol–ethyl acetate–water (7:1:2); and II, ethanol (95%)–ammonium formate (0.5M) (7:3).

Paper electrophoresis was performed in a buffer at pH 3.8 that was 0.034M in ammonium formate and 0.078M in formic acid.

IV. Liquid-scintillation spectrometry. — All liquid-scintillation spectrometry was carried out in a Packard Tri-Carb Scintillation Spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Quenching was corrected for by use of the external standard. Aqueous samples of up to 2.0 ml were assayed in the presence of 10 ml of the following mixture: toluene (800 ml), Triton X-100 (200 ml), and Omnifluor (4.0 g). Samples of nicotinamide were dissolved and also counted in 10 ml of this fluid. *p*-Phenylphenacyl lactate was assayed for radioactivity in 10 ml of Bray's fluid[12].

RESULTS

To show that the C-6 hydrogen atoms of UDP-Glc are transferred directly to NAD, UDP-Glc-*6-t* was incubated with homogeneous beef-liver UDP-Glc dehydrogenase in the presence of NAD, as described in the Methods section. When the reaction was complete, the NADH was completely oxidized by the addition of pyruvic acid and lactic acid dehydrogenase. According to Krakow *et al.*[7] the initial hydrogen transfer occurs to the B side of the nicotinamide ring. If both C-6 hydrogen atoms are transferred directly to NAD during this reaction, subsequent oxidation of the NADH with lactic acid dehydrogenase should not remove them, since lactic acid dehydrogenase exhibits A stereospecificity towards NADH[13].

In order to demonstrate the presence of the C-6 hydrogen atoms in NAD, a portion of the reaction mixture was hydrolyzed to liberate free nicotinamide. Table I

shows that all the recoverable radioactivity was found in nicotinamide, and there was no transfer of radioactivity to either lactic acid or the reaction medium.

TABLE I[a]

TRANSFER OF THE C-6 HYDROGEN ATOMS OF UDP-Glc-*6-t* TO NAD DURING THE ENZYMIC CONVERSION OF UDP-Glc INTO UDP-GlcA

Substance	*% of Radioactivity initially in UDP-Glc-6-t*	
	Reaction	*Control*
Nicotinamide	85.1	<0.2
Lactic acid	<0.2	<0.2
Water	<0.2	<0.2

[a]Enzymic reactions were carried out as described under Methods. The nicotinamide recovered was thrice crystallized, and aliquots (29.5–30.7 mg) of the second and third crops (m.p. 128–129°) were assayed for radioactivity as described. The corresponding specific radioactivities agreed within 1%. Lactic acid was converted into its *p*-phenylphenacyl derivative, which was crystallized four times as described. Aliquots (10.1–12.0 mg) of the third and fourth crop of crystals (m.p. 150–151°) were assayed for radioactivity. The water referred to was recovered from the reaction and control media by lyophilization. All samples were counted for 200 min.

From these results we conclude that both C-6 hydrogen atoms of UDP-Glc are transferred directly to NAD during the enzymic conversion of UDP-Glc into UDP-GlcA by UDP-Glc dehydrogenase. In addition, the B stereospecificity of the reduction of NAD, previously noted by Krakow *et al.*[7], is confirmed.

DISCUSSION

In order to design experiments to elucidate the details of the mechanism of action of UDP-Glc dehydrogenase, one must establish the origin and fate of the atoms involved in the reaction.

The first mechanistic studies were carried out by Krakow *et al.*[7], who demonstrated that, in the dehydrogenation of UDP-Glc, transfer of hydrogen to NAD occurs with B stereospecificity. However, the source of the hydrogen atoms transferred was undefined. On the basis of results obtained with impure bovine UDP-Glc dehydrogenase, Simonart *et al.*[14] suggested that the reaction proceeds through an intermediate in which the uracil moiety has been oxidized. However, Zalitis and Feingold[15] could not confirm these results with pure beef-liver enzyme, and were unable to demonstrate the intermediate. About this time Wang and Bartnicki-Garcia[16] showed that 96% of the tritium from UDP-Glc-*6-t* was released to the medium during its oxidation with impure, commercial, beef-liver dehydrogenase, implying that transfer of hydrogen from C-6 of UDP-Glc to NAD is not direct but rather proceeds through an intermediate from which hydrogen exchange with water could occur. This finding con-

tradicted those for other dehydrogenases previously investigated. The results of our experiments with homogeneous beef-liver enzyme show that, during the course of the reaction, the C-6 hydrogen atoms of UDP-Glc are transferred directly to NAD with no loss to the reaction medium. Furthermore, we were able to confirm the B stereospecificity of hydrogen transfer initially demonstrated by Krakow *et al.*[7].

REFERENCES

1 G. J. DUTTON AND I. D. E. STOREY, *Biochem. J.*, 48 (1951) XXIX.
2 J. L. STROMINGER, H. M. KALCKAR, J. AXELROD, AND E. S. MAXWELL, *J. Amer. Chem. Soc.*, 76 (1954) 6411.
3 G. J. DUTTON, in G. J. DUTTON, (Ed.), *Glucuronic Acid Free and Combined*, Academic Press, New York, 1966, Ch. III.
4 H. ANKEL, E. ANKEL, AND D. S. FEINGOLD, *Biochemistry*, 5 (1966) 1864.
5 A. BDOLAH AND D. S. FEINGOLD, *J. Bacteriol.*, 96 (1968) 1144.
6 M. M. LIEBERMAN, A. SHAPIRAS, AND A. MARKOWITZ, *J. Bacteriol.*, 101 (1970) 959.
7 G. KRAKOW, J. LUDOWIEG, J. H. MATHER, W. M. NORMORE, L. TOSI, S. UDAKA, AND B. VENNESLAND, *Biochemistry*, 2 (1963) 1009.
8 A. WRIGHT AND P. W. ROBBINS, *Biochim. Biophys. Acta*, 104 (1965) 594.
9 J. ZALITIS AND D. S. FEINGOLD, *Arch. Biochem. Biophys.*, 132 (1969) 457.
10 A. I. VOGEL, *A Textbook of Practical Organic Chemistry*, Longmans, Green and Co., London, 1948, p. 359.
11 S. P. COLOWICK, N. O. KAPLAN, AND M. M. CIOTTI, *J. Biol. Chem.*, 191 (1951) 447.
12 G. A. BRAY, *Anal. Biochem.*, 1 (1960) 279.
13 F. A. LOEWUS, P. OFNER, H. F. FISHER, F. H. WESTHEIMER, AND B. VENNESLAND, *J. Biol. Chem.*, 202 (1953) 699.
14 P. C. SIMONART, W. L. SALO, AND S. KIRKWOOD, *Biochem. Biophys. Res. Comm.*, 24 (1966) 120.
15 J. ZALITIS AND D. S. FEINGOLD, *Biochem. Biophys. Res. Comm.*, 31 (1968) 693.
16 M. C. WANG AND S. BARTNICKI-GARCIA, *Anal. Biochem.*, 26 (1968) 412.