THE STEREOSPECIFICITY OF HYDROGEN ABSTRACTION BY URIDINE DIPHOSPHOGLUCOSE DEHYDROGENASE

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SUMMARY: UDP-glucose dehydrogenase catalyzes the incorporation of tritium into UDP-glucose (UDPG) in the presence of UDP-α-D-gluco-hexodialdose (UDP-Glc-6-CHO) and [B-^{3}H]-NADH. The ^{3}H is located exclusively at C-6 of the glucose moiety of UDPG and at least 79% of it is in the pro-R position. It is concluded that UDPG dehydrogenase catalyzes the abstraction of the pro-R hydrogen at C-6 of the glucose moiety of the substrate as the first step in the conversion of UDPG to UDP-glucuronic acid. The apparent lack of complete stereospecificity has been shown to result from a hitherto undetected reversible redox reaction prior to the release of UDP-glucuronic acid by the enzyme.

UDPG dehydrogenase from bovine liver catalyzes the formation of UDP-glucuronic acid (UDP-GlcUA) from UDPG and Nelsestuen and Kirkwood (1) have shown that this reaction involves at least two steps with UDP-Glc-6-CHO serving as an enzyme-bound intermediate. Since a C-6 hydrogen atom is removed in the first step, it is of interest to determine if this abstraction is stereospecific. We wish to report data establishing that UDPG dehydrogenase catalyzes the stereospecific abstraction of the pro-R hydrogen in the conversion of the C-6 carbinol of UDPG to the C-6 aldehyde of UDP-Glc-6-CHO. In the course of these investigations we have detected a second reversible redox step in the enzyme mechanism.

MATERIALS AND METHODS

Enzymes and chemicals: UDPG dehydrogenase was purified from bovine liver by the method of Straw (2). The specific activity of the enzyme preparation used in this work was 2.8 i.u. per mg of protein.

Glucose dehydrogenase and α-glycerophosphate dehydrogenase were obtained from Sigma. Glycerol kinase, pyruvate kinase, rabbit muscle aldolase, and crystalline triosephosphate isomerase were obtained from Calbiochem. The commercial preparation of aldolase was dialyzed against 1mM EDTA, pH 7.4, for 6 hours. Aldolase treated in this manner contained no detectable isomerase activity when the latter was assayed according to Norton et al. (3). The commercial isomerase preparation was dialyzed for 6 hours against 1mM Tris-HCl, pH 7.4, and following this treatment aldolase contamination was reduced to less than $1.8 \times 10^{-4}\%$, on an activity basis, when assayed by the method of Hartman (4). In this communication the unitage systems used for all enzymes directly from commercial sources were those of the suppliers. The units and mode of assay for aldolase was that of Hartman (4) and those for isomerase were as described by Norton et al. (3). Glucose-1-^{3}H (3 Ci per mmole) was the product of International Chemical and Nuclear Corp. and phenazine methosulfate was purchased from Nutritional Biochemicals Corp.

Synthesis of [B-^{3}H]-NADH: Radioactively labeled [B-^{3}H]-NADH was synthesized from glucose-1-^{3}H and NAD$^+$ using glucose dehydrogenase (a B-specific enzyme (5)) from bovine liver. The tritium-labeled NADH was purified by chromatography on DEAE-cellulose column (HCO_3-) eluted with an NH_4HCO_3 gradient, pH 7.0. The specific activity of the [B-^{3}H]-NADH was 1.41×10^8 DPM/µmole and the yield was 77%.

RESULTS AND DISCUSSION

Enzymatic reduction of UDP-Glc-6-CHO with [B-^{3}H]-NADH: UDP-Glc-6-CHO was synthesized by the method of Nelsestuen and Kirkwood (1). UDP-Glc-6-CHO (0.14 µmoles) was incubated at room temperature for 5 hours with 0.26 µmoles of [B-^{3}H]-NADH (1.41×10^8 DPM/µmole), 85 µmoles of glycine buffer pH 8.7, and 75 mi.u. of UDPG dehydrogenase in a total volume of 0.25 ml. The reaction mixture, together with 1 µmole of UDPG as a carrier, was purified on a DEAE-cellulose column (HCO_3-) by irrigating with a linear gradient from zero to 0.12 M NH_4HCO_3, pH 7.0. The UDPG fractions were pooled, and further purified by paper chromatography using

1 M ammonium acetate (pH 7.5)-absolute ethanol (1:3)(6). Rechromatography on DEAE-cellulose yielded a radiochemically chromatographically pure sample of tritiated UDPG (1.3×10^6 DPM). A control incubation lacking enzyme incorporated no label into UDPG.

The site of enzymatic reduction of UDP-Glc-6-CHO with [B-^{3}H]-NADH: Glucose was released from the sample by acid hydrolysis (0.2 N HCl, 20 min at 100°) and the glucose purified and isolated by paper chromatography in pyridine-ethyl acetate-water (2:5:7)(7). A portion of the glucose eluted from the paper (1.7×10^4 DPM) was oxidized with $NaIO_4$ in the presence of carrier ethylene glycol. The resulting formaldehyde was precipitated as its methone derivative and proved to have a constant specific activity over three recrystallizations which was within experimental error of that calculated from the specific activity of the starting glucose. This indicates that all of the ^{3}H is attached to C-6 of the glucose.

Degradation of glucose-6-^{3}H: The stereochemistry of the ^{3}H label in the UDPG was related to the known absolute stereospecificities of fructose-1, 6-di-P aldolase and triose-P-isomerase through conversion of C-4, 5 and 6 of the glucose moiety into dihydroxacetone-P, a substrate for both enzymes.

a. Isolation of C-4, 5 and 6 as glycerol: The method used was an adaptation of the scheme described by Bevill *et al.* (8). Alpha-methyl glucoside synthesized from glucose-6-^{3}H (6.52×10^5 DPM) was oxidized with $NaIO_4$, and the resulting dialdehyde reduced with $NaBH_4$ and then hydrolyzed with 1 N HCl to yield ^{3}H-glycerol from C-4, 5 and 6 of the original glucose-6-^{3}H. The glycerol was purified by paper chromatography in ethyl acetate-pyridine-water (12:5:4) and its yield, based on α-Me-glucoside-6-^{3}H, was 48%.

b. Synthesis of L-[1-^{3}H]-glycerol-3-P: ^{3}H-glycerol (5.16×10^5 DPM), 30 μmoles redistilled carrier glycerol, 0.22 mmoles of Tris-HCl buffer pH 7.4, 45 μmoles of neutralized ATP, 9 μmoles of $MgCl_2$ and

Table 1. Stereochemistry of the ^3H in ^3H-dihydroxyacetone-P (^3H-DHAP).

Experiment	Recovery of Counts (%)	Recovered DPM in water wash (%)	Recovered DPM Retained by a Dowex-1-Cl Column [%]
1. Aldolase (exchanges pro-S)	89.1	20.9	79.1
2. Isomerase (exchanges pro-R)	81.1	94.4	5.6
3. Control (no enzyme)	88.5	12.0	88.0

^3H-DHAP (1.67 micromoles, 4.05 X 10^3 DPM/micromole) and 50 mmoles of Tris-HCl buffer, pH 7.5 were incubated for 4 hours at 28° with either aldolase (32.1 units), isomerase (330 units), or without enzyme (control) in a total volume of 0.8 ml. Anions were removed by passage through a Dowex-1-Cl column (2 X 3 cm) followed by a 50 ml water wash. DHAP was eluted from the column with 0.05 N HCl (50 ml) and the DPM present in the two fractions were determined.

2.5 mg of bovine plasma albumin were reacted with 16 units of glycerol kinase in a total volume of 2.06 ml for 4 1/4 hours at 37°. An ATP regenerating system consisting of 22 μmoles of phosphoenol pyruvate and 26 units of pyruvate kinase was added to increase the yield of α-glycerol-P. The reaction was stopped by heating for 30 seconds in boiling water bath and, following centrifugation, the supernate was adjusted to pH 7.0 and transferred to a 1 x 14 cm column of Dowex-1-formate. The column was irrigated with a 2 liter HCOOH gradient (0 to 1 N) and the α-glycerol-P was isolated and the HCOOH extracted with ether (9). The α-glycerol-P was further purified at pH 3.0 with acid washed Norit A charcoal. Yield: 54%.

c. <u>Synthesis of [1-^3H]-Dihydroxyacetone-3-P (^3H-DHAP):</u> ^3H-DHAP was synthesized in a reaction mixture containing L-[1-^3H]-glycerol-3-P (18-20 μmoles), NAD^+ (10 μmoles), EDTA (6 μmoles), Tris-HCl pH 7.4 (90 μmoles), 95% ethanol (0.05 ml), 2 x crystallized catalase (Schwarz/

Mann, 950 units), and glycerol-P dehydrogenase (30 units) in a total volume of 2.9 ml. The reaction was started by the addition of 0.1 ml of a phenazine methosulfate solution (2 mg/ml) and air was slowly bubbled through the mixture. After a 10 hour incubation, the phenazine methosulfate was removed with IR-120 (H^+), the solution was diluted to 20 ml, neutralized to pH 7.0 and the products were separated by Dowex-1-formate column chromatography as described previously for glycerol phosphate. Under these conditions α-glycerol-P is well separated from DHAP. Yield of ^{3}H-DHAP was 21%.

Stereochemistry of the tritium at C-6 of the glucose moiety of UDPG: The ^{3}H-DHAP isolated from glucose-6-^{3}H by the degradation scheme outlined previously is labeled with tritium only in the hydroxymethyl group. Fructose-1,6-di-P aldolase in the absence of aldehyde acceptor exchanges the pro-S hydrogen of the hydroxymethyl group of DHAP with the protons of the medium (10). Triose-P-isomerase has been shown to have an absolute stereospecificity opposite to that of fructose-1,6-di-P aldolase and exchanges the pro-R hydrogen (10). Both aldolase and isomerase were utilized in separate experiments to provide a double check of the absolute stereospecificity. The conditions and results of these experiments are shown in Table 1.

In the presence of aldolase 21% of the DPM recovered appeared in the aqueous medium indicating that 21% of the original label was in the pro-S position at C-1 of DHAP. Based on these data one would have predicted that 79% of the original label was in the pro-R position and should appear in the medium when ^{3}H-DHAP was incubated in the presence of isomerase. Experiment 2 in Table 1 shows that in the presence of isomerase 94% of the original label was exchanged. This discrepency is due to the slight residual contamination of the isomerase preparation with aldolase activity as noted in the Materials and Methods section. In the absence of either enzyme only a small amount of tritium (12%) appeared in the medium.

These data indicate that the first step in the conversion of UDPG to UDP-GlcUA by UDPG dehydrogenase is the abstraction of the pro-R hydrogen at C-6 of the glucose moiety of the substrate. The apparent lack of complete stereospecificity indicated by the experimental results is undoubtedly due to the reversibility of the second oxidation step in the enzyme mechanism. It has been assumed in the past that this step involved the oxidation of the 6-aldehyde to the 6-carboxylic acid, through the intervention of a second mole of NAD^+. Such a reaction would be irreversible and would conveniently account for the well established irreversibility of the overall reaction. However, we have recently shown (11) that prolonged incubations of UDPG in the presence of $[B\text{-}^3H]$-NADH and only catalytic quantities of NAD^+ leads to equivalent labeling of the pro-R and pro-S hydrogens. This indicates that the oxidation of the 6-carboxylic acid is a reversible process and thus must involve a derivative of the acid, perhaps a thiol ester. It is the hydrolysis of this derivative, then, that accounts for the overall irreversibility of the process. Since it has not yet been possible to determine the stereochemistry of the first step in the complete absence of the operation of the second, some labeling of both hydrogens must occur in the course of the experimental procedure.

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