EVIDENCE FOR URIDINE 5'-(α-D-GALACTOPYRANOSYL PYROPHOSPHATE):NAD 2-HEXOSYL OXIDOREDUCTASE IN PENICILLIUM CHARLESII

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SUMMARY

A heat labile fraction from Penicillium charlesii was isolated which catalyzes the oxidation of UDP-galactose in the presence of NAD+ with the formation of NADH and a 2-keto sugar presumably attached to UDP. A portion of this sugar is in the furanosyl form. A mechanism for conversion of the pyranosyl to furanosyl ring form is proposed and UDP-2-ketogalactopyranoside and UDP-2-ketogalactofuranoside are suggested as intermediates in the conversion of uridine 5'-(α-D-galactopyranosyl pyrophosphate) to uridine 5'-(α-D-galactofuranosyl pyrophosphate).

INTRODUCTION

At present little is known about the biosynthesis of galactofuranosides and the reaction in which the pyranosyl-furanosyl isomerization occurs is unknown. Trejo et al. (1) have isolated uridine 5'-(α-D-galactofuranosyl pyrophosphate), UDP-Gal_f, from Penicillium charlesii. Sarvas and Nakaido (2) have presented evidence that uridine 5'-(α-D-galactopyranosyl pyrophosphate), UDP-Gal_p is a precursor of the galactofuranosyl residues in the O-antigen region of lipopolysaccharide obtained from a T1 mutant of Salmonella typhimurium. Presumably UDP-Gal_p is a precursor of UDP-Gal_f. We have preliminary evidence (3) showing that a UDP-Gal_p 4-epimerase negative mutant of P. charlesii synthesizes the peptidomannan portion of a glycopeptide which contains galactofuranosyl and mannopyranosyl residues when isolated from the parent organism. This evidence (2, 3) indirectly suggests that UDP-Gal_p is on the galactofuranosyl biosynthetic pathway and that of Trejo et al. (1) establishes UDP-Gal_f as a galactofuranosyl donor in glycopeptide biosynthesis.

MATERIALS AND METHODS

a. Enzyme preparation

Penicillium charlesii was grown as a stationary culture for 3 days (4). The

fungal mats were removed from the growth medium and washed with distilled H_2O. 12 g of fungi were macerated in 0.05 M tris-HCl:0.3 M NaCl pH 8.3 containing 10 mM dithiothreitol and 12 g Al_2O_3 previously washed with EDTA. The paste was centrifuged at 27,000 x g at 0^o for 30 min. The supernatant solution was removed and 2 mg protamine sulfate (Sigma Chemical Co.) was added per ml of solution. The mixture was stirred gently for 30 min and centrifuged at 27,000 x g at 0^o for 30 min. The supernatant solution was fractionated with saturated $(NH_4)_2SO_4$ containing approximately 5 mM dithiothreitol and the material precipitating between 40% and 55% saturated $(NH_4)_2SO_4$ was used as the enzyme preparation. The 6-fold purified enzyme preparation contained 0.78 unit of enzymic activity/mg of protein. 1 unit of enzyme catalyzes the reduction of 1 µmole of NAD^+/ min in a system containing NAD^+ and UDP-Gal_p.

b. Enzyme assay

Enzyme assays contained the following unless otherwise specified: 0.05 M tris-HCl:0.3 M NaCl:10 mM dithiothreitol, 485 µl; NAD^+, 1 µmole; UDP-Gal_p, 1 µmole; enzyme preparation, 0.1 unit of activity; total volume 500 µl. The rate of the reaction was monitored spectrophotometrically at 340 nm.

c. Paper chromatography

The products of the reactions containing either [^{3}H]- or [^{14}C]-labeled UDP-galactose and its derivatives were separated by descending chromatography on Whatman 1 paper using ethanol:1 M ammonium acetate, pH 7.5 (7:3, v/v) as the solvent.

d. Preparation of derivatives and degradation products of galactose

Potassium galactonate, the benzimidazole derivative formed by reaction of galactonate with o-phenylenediamine, 2-benzimidazole aldehyde and 2-benzimidazole carboxylic acid were prepared as described by Bernstein et al. (5). The galactonate and benzimidazole derivative were recrystallized twice. Treatment of 2-benzimidazole aldehyde with alkaline $KMnO_4$ releases the hydrogen atom from the aldehyde group. The H released was distilled from an alkaline solution and the ^3H estimated in a scintillation spectrometer. Under these conditions formate is

not distilled and formaldehyde, released by periodate oxidation of the benzimidazole derivative, contained no ^{3}H.

Formaldehyde, obtained by treating galactitol or UDP-galactose derivatives with $NaIO_4$ after adding 89.5 μmoles of ethylene glycol, was trapped as the dimedon derivative (6). The $NaIO_4$ treatment was carried out for 1 hr at pH 8.0 and excess $NaIO_4$ destroyed with $NaAsO_2$. The dimedon derivative was recrystallized to constant specific activity. The quantity of ^{3}H was estimated on a weighed sample in a liquid scintillation spectrometer.

RESULTS

Incubation of UDP-Gal_p with NAD^+ and an enzyme fraction obtained from P. charlesii resulted in a time dependent increase in absorbance at 340 nm. Substitution of either UDP-glucose or α-D-galactopyranosyl 1-phosphate for UDP-Gal_p or NADP for NAD^+ resulted in no change in absorbance at 340 nm. Furthermore, the initial velocity of the reaction was linear with respect to enzyme concentration over the 10-fold range of concentrations tested. Apparent K_m's for NAD^+ and UDP-Gal_p of 0.7 mM and 1 mM respectively were obtained.

Experiments were conducted to determine if UDP-Gal_p becomes oxidized during the course of the reaction. Following incubation of UDP-[^{14}C]-Gal_p (2.0 μmoles) with NAD^+ (2.0 μmoles) and 0.1 unit of enzyme for 30 min the reaction mixture was chromatographed. The major ^{14}C peak was coincident with UDP-Gal_p. An additional peak containing 10% of the total ^{14}C migrated at an $R_{UDP\text{-}Gal_p}$ of 1.2. Uridine 5'-(α-D-galacturonopyranosyl pyrophosphate), NAD^+ and α-D-galactopyranosyl 1-phosphate migrate at $R_{UDP\text{-}Gal_p}$ values of 0.5, 0.7 and 0.9 respectively in this solvent. We conclude that the product of the reaction is not UDP-galacturonic acid, and that the product could be uridine 5'-(2-, 3-, or 4-ketogalactopyranosyl pyrophosphate). Each of these products would, upon treatment with alkaline [^{3}H]-$NaBH_4$ be reduced to UDP-Gal_p and an isomeric UDP-hexose.

UDP-Gal_p (20 μmoles) was incubated with NAD^+ (20 μmoles) and 1 unit of enzyme at room temperature for 30 min. Approximately 1.4 μmoles of NADH was formed. The reaction products were chromatographed, the ultraviolet-quenching area of the

chromatogram and the region immediately ahead of it to include the substance migrating at $R_{UDP\text{-}Gal_p}$ of 1.2 was removed and the paper irrigated with H_2O. The eluate was concentrated and treated at room temperature for 8 hr with alkaline (pH 10.5) [^{3}H]-$NaBH_4$. The reaction mixture, after decomposing the unreacted $NaBH_4$ was chromatographed. The radioactive area of the chromatogram ($R_{UDP\text{-}Gal_p}$ of 1.1) was removed and irrigated with H_2O. The eluate contained approximately 50% of the quantity of ^{3}H expected on the basis of μmoles UDP-Gal_p oxidized; a quantity well within the error of weighing $NaBH_4$ and the manufactures variability in specific activity. The eluate was treated with 1.2 unit of alkaline phosphatase at room temperature and following chromatography the ^{3}H now appeared as one peak, $R_{UDP\text{-}Gal_p}$ of 1.7. This migration rate is approximately equivalent to that of galactose. No ^{3}H was observed in the region where talose, the 2-epimer of galactose, migrates.

The radioactive region of the chromatogram was removed, irrigated with H_2O and the solution concentrated. D-Galactose (1.1 mmoles) was added to an aliquot of the solution and potassium galactonate was prepared. The aldonate was then converted to the benzimidazole derivative of galactose and thence to 2-benzimidazole aldehyde. Table 1 shows the specific activities of each derivative. Tt shows that about 5% of the ^{3}H was associated with carbon atom 1, 73% with carbon atom 2 and the remainder with one or more of carbon atoms 3 through 6. In an independent experiment in which [^{3}H]-galactose was reduced to galactitol and this in turn treated with $NaIO_4$ and the formaldehyde released was isolated as the dimedon derivative, it was shown that 5.4% of the ^{3}H was located in C-1 and C-6 atoms combined. More important, treatment of 2-benzimidazole aldehyde (4.6×10^4 dpm) with $KMnO_4$ resulted in the release of ^{3}H (4.05×10^4 dpm) which distilled over at 100° from alkaline solution. These data suggest that the majority of the ^{3}H is attached to carbon atom 2.

These data do not indicate whether the 2-ketogalactose derivative is all in the pyranosyl form or a mixture of pyranosyl and furanosyl forms. An experiment was conducted to determine if the enzyme preparation also catalyzed a pyranosyl-

Table 1

LOCATION OF ^{3}H GALACTOSE[a]

	quantity (mmole)	total ^{3}H (dpm)	spec. act. (dpm/mmole)
Aldose initially[b]	1.1	13.6×10^{5}	12.3×10^{5}
K-Galactonate	0.64	7.45×10^{5}	11.7×10^{5}
Benzimidazole derivative	0.044	5.3×10^{4}	11.9×10^{5}
]-Benzimidazole aldehyde	0.018	1.65×10^{3}	8.92×10^{5}

[a] One unit of enzyme was incubated with UDP-Gal_p (20 µmoles) and NAD^{+} (20 µmoles) for 30 min. Following paper chromatography the uridine-containing reaction products were eluted and the eluate treated with [^{3}H]-$NaBH_4$ at pH 10.5. The unreacted $NaBH_4$ was decomposed and the reaction mixture chromatographed on paper. A radioactive area, $R_{UDP\text{-}Gal_p}$ of 1.1 was cut from the paper and irrigated with H_2O. The eluate was treated with alkaline phosphatase, the reaction mixture again chromatographed and the radioactive area of the chromatogram irrigated with H_2O. Five µl from 0.5 ml contained 13.6×10^{5} dpm.

[b] D-galactose (1.1 mmoles) was added to the [^{3}H]-product eluted from the chromatogram and the various derivatives were prepared.

furanosyl isomerization reaction. Treatment of UDP-[6-^{3}H]-Gal_p or a pyranosyl derivative with $NaIO_4$ should not result in the release of [^{3}H]-formaldehyde. In contrast, treatment of UDP-[6-^{3}H]-Gal_f with $NaIO_4$ should result in the release of [^{3}H]-formaldehyde. In this experiment the reaction mixture contained UDP-[6-^{3}H] Gal_p (0.2 µmole), NAD^{+} (2 µmoles) and 0.1 unit of enzyme. The reaction proceeded until 0.17 µmole of NADH was formed; presumably only 0.03 µmole of UDP-Gal_p remained. The components were chromatographed and ^{3}H was found in two adjacent positions (areas A and B) on the chromatogram; $R_{UDP\text{-}Gal_p}$ of 1.0 and 1.2. These areas were removed, irrigated with H_2O, the solutions treated with $NaIO_4$ and the formaldehyde precipitated as the dimedon derivative. Table 2 shows that approximately 14% of the ^{3}H in area A was recovered in the dimedon and that only 3.5% of that in area B was in the dimedon. Thus it appears that region B contains the 2-ketogalactopyranosyl derivative while area A contains both UDP-Gal_p and the 2-ketogalactofuranosyl derivative.

Table 2

RELEASE OF [^{3}H]-FORMALDEHYDE FROM

UDP-[6-^{3}H]-D-GALACTOSE DERIVATIVES BY $NaIO_4$

	Position on Chromatogram	
	A	B
Initial ^{3}H eluted (dpm)	2.2×10^4	1.75×10^5
Dimedon, specific activity (dpm/mg)	8.7×10^1	3.18×10^2
Total ^{3}H in dimedon[a] (dpm)	4.5×10^3	1.66×10^4
% of ^{3}H released as formaldehyde[b]	14	3.5

[a] Based on a value of 52.2 mg dimedon from 89.5 μmoles of ethylene glycol and [^{3}H]-product.

[b] Corrected for [^{3}H]-dimedon from a sample of UDP-[6-^{3}H]-Gal_p. The enzyme preparation (0.1 unit) was incubated with UDP-[6-^{3}H]-Gal_p (0.2 μmole) and NAD^+ (2.0 μmoles) until there was no further increase in absorbance at 340 nm. The reaction mixture was chromatographed, and 2 ^{3}H-containing areas of the chromatogram were irrigated with H_2O. After adding 89.5 μmoles ethylene glycol to each eluate the solutions were treated with excess $NaIO_4$ at pH 8 for 1 hr. The excess $NaIO_4$ was destroyed with $NaAsO_2$. The formaldehyde released was isolated as the dimedon derivative and recrystallized to constant specific activity.

DISCUSSION

The results of these experiments suggest that P. charlesii extracts contain an enzyme which catalyzes the conversion of UDP-Gal_p to a 2-ketogalactose derivative, possibly UDP-2-ketogalactose, with NAD^+ as the electron acceptor. The small quantity of ^{3}H found attached to carbon atoms 1, 3, 4 or 5 probably results from migration of the double bond under alkaline conditions during [^{3}H]-$NaBH_4$ reduction.

Mendicino and Hanna (7) noted that apiofuranosyl 1-phosphate and presumably UDP-apiofuranoside, readily undergo hydrolysis to apiofuranosyl 1,2-cyclic-phosphate. It is feasible that during reduction with [^{3}H]-$NaBH_4$ the alkaline conditions promoted hydrolysis of the UDP-hexose to galactose 1,2-cyclic-phosphate

or galactose 2-phosphate. Treatment with alkaline phosphatase resulted in the formation of a substance with the properties of galactose. No ^{3}H was found in the 2-epimer of galactose, talose. The hydroxyl groups attached to the C-3 and C-4 atoms apparently influence the stereospecificity of the reduction of the 2-ketogalactose derivative.

The data do not allow a precise estimate of the fraction of the oxidized sugar in the pyranosyl and furanosyl forms. However, the data do suggest that a portion of the nucleotide was isomerized to the furanosyl configuration. It is proposed that reactions 1 and 2 below constitute the first 2 reactions in the conversion of UDP-Gal_p to UDP-Gal_f. There is no evidence to suggest that this preparation also catalyzes the reduction of UDP-2-ketogalactofuranoside to UDP-Gal_f.

$$\text{UDP-Gal}_p + \text{NAD}^+ \rightleftarrows \text{UDP-2-keto-Gal}_p + \text{NADH} + \text{H}^+ \qquad (1)$$

$$\text{UDP-2-keto Gal}_p \rightleftarrows \text{UDP-2-keto Gal}_f \qquad (2)$$

Molecular models of α-D-2-ketogalactopyranose show that in one boat conformation the C-4 hydroxyl group is in close proximity to the C-1 atom. Thus, oxidation at the C-2 atom appears to sterically promote the movement of the C-4 hydroxyl group nearer to the C-1 atom. This attraction of the hydroxyl group is enhanced by a slight positive charge on both C-1 and C-2 atoms. Pyranosyl to furanosyl ring contraction should be facilitated by removal of the proton from the C-4 hydroxyl group and simultaneous protonation of the pyran ring oxygen. The furan ring of the 2-ketofuranoside is stabilized in a near planar conformation. In this conformation the furanosyl-pyranosyl isomerization can occur by a slight rotation of the glycol group attached to the C-4 atom. Therefore, the proposed UDP-2-keto Gal_p and UDP-2-keto Gal_f are attractive intermediates in the isomerization of UDP-Gal_p to UDP-Gal_f.

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