The Synthesis of Guanosine 5'-Diphosphate L-Fucose from Guanosine 5'-Diphosphate 3,5-d-[3H]Mannose Catalyzed by an Enzyme Extract from Fruits of the Flax

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ABSTRACT

An enzyme system from fruits of the flax plant is described that catalyzes the synthesis of the sugar nucleotide guanosine 5'-diphosphate L-fucose from guanosine 5'-diphosphate D-mannose with the intermediate formation of guanosine 5'-diphosphate 4-keto-6-deoxy-D-mannose. Tritium from [3H]H2O was incorporated into the L-fucose portion of the sugar nucleotide in the course of the reaction, and tritium at the 3,5-carbons of the D-mannose moiety of GDP-D-mannose was exchanged with protons in the medium. These results support a mechanism of synthesis analogous to that proposed for the formation of L-rhamnose and other 6-deoxy sugars.

The flax plant (Linum usitatissimum) synthesizes and stores in its seed large amounts of a H2O-soluble, mucilaginous polysaccharide. About 25% of that molecule is made up of L-galactose in a chain which also includes D-galacturonic acid, L-rhamnose, and D-xylene (1). It seemed likely, therefore, that the fruits or leaves of that plant would be a good source of the enzyme that synthesizes L-galactose, presumably via the sugar nucleotide derivative. Although small amounts of guanosine 5'-diphosphate L-galactose were formed from guanosine 5'-diphosphate D-mannose when that compound was incubated with enzyme extracts of flax fruits (2), a much greater conversion of the substrate to guanosine 5'-diphosphate L-fucose was observed when NADPH was included in the reaction mixtures. Why this particular system predominates in the cell-free enzyme preparations is not clear. It seems likely, however, that, at some time during the development of the flax fruit, a complex carbohydrate is produced containing L-fucose for which that sugar nucleotide serves as the glycosyl donor.

Here, the preparation of the flax fruit extract that catalyzes the synthesis of L-fucose and some of the properties of that reaction are described. In addition, results of experiments with the flax enzymes and [3H]-labeled substrates are reported which support the chemical pathways proposed for the conversion of GDP-D-mannose to GDP-L-fucose.

EXPERIMENTAL PROCEDURES

Materials and Methods. GDP-D-mannose labeled uniformly with 14C in the D-mannosyl moiety (about 150 μCi/μmol) was obtained from the Amersham Corp. as was also tritium-labeled H2O (90 μCi/μmol). Unlabeled sugar nucleotides were obtained from Sigma or P-L Biochemicals. Authentic GDP-β-L-fucose was kindly provided by Dr. Robert Barker. All other reagents were obtained from the usual commercial sources.

Paper electrophoresis was performed on a flat plate apparatus as designed by Crestfield and Allen (5) at about 40 v/cm in 100 mm ammonium formate buffer (pH 2.7) with Schleicher and Schuell No. 589 orange ribbon paper. Desiccated paper chromatography was carried out on Schleicher and Schuell No. 589 white ribbon paper in 15- x 45-cm cylindrical tanks. Solvent systems used were: I, 1-propanol-ethyl acetate-water (7:1:2, v/v); II, 95% ethanol, 1.0 M ammonium acetate (7.3, v/v). Radioactivity was measured in a Beckman model LS-230 scintillation spectrometer in a mixture consisting of 1 ml H2O containing the sample and 10 ml 33% Triton, 0.01% 1,4-bis(2-[5-phenyloxazolyl])benzene, and 0.4% 2,5-diphenyloxazole in toluene. When labeled material on chromatography or electrophoresis papers was to be counted, the appropriate areas of the paper, located by the movement of adjoining standard compounds, were cut out and eluted by shaking in 1 ml H2O in a scintillation vial for 15 min.

Synthesis of GDP-D-Mannose Labeled with 3H at the 3,5-Carbons of the D-Mannosyl Moiety. This compound was synthesized enzymically and characterized in the course of a study on the mechanism of the enzymic epimerization of GDP-D-mannose to GDP-L-galactose (3). The preparation actually consists of about 20% 3H-labeled GDP-L-galactose and 80% 3H-labeled GDP-D-mannose. It was obtained by incubating GDP-D-mannose in [3H]H2O with an enzyme from the green alga Chlorella pyrenoidosa. It had been demonstrated that the reversible epimerization of GDP-D-mannose to GDP-L-galactose in that system is accompanied by the exchange of hydrogen with the medium at those carbons where epimerization occurs (3).

Growth of Flax and Preparation of the Enzyme System that Catalyzes the Synthesis of L-Fucose. Flax seeds were obtained from a local health food store and the plants were grown outdoors during the summer months of 1978 and 1979 in garden space provided and cared for by Dr. Jack Beal and his staff of the College of Pharmacy. The fruits were harvested by hand after 8 to 10 weeks. They were picked a few days after fertilization when they were still green and undescended. Only the fruits that were yellow-green (at this stage they had an orange color) and about 5 mm in diameter were used. They were chilled on ice and used that day or were frozen and stored in the freezer at −15°C where they seemed to lose little or no activity over a year or more. Mysteriously, the activity of one batch that was frozen and stored at −70°C was much lower than that observed in fruits stored at the higher temperature.

A typical enzyme extract was prepared as follows. Flax fruits after being thawed (26 g) were washed and chilled on ice, and all subsequent operations were conducted in the cold. They were ground in a mortar with acid-washed sand in 100 ml 100 mm sodium-potassium phosphate buffer (pH 6.9) with 1 mm disodium EDTA, 25 mM β-mercaptoethanol, and 4% soluble PVP (Arthur Thomas Co.). The homogenate was squeezed through four layers
of cheesecloth and centrifuged for 20 min at 20,000g to remove particulate material. The protein that precipitated between 33 and 50% saturation with ammonium sulfate was collected by centrifugation, suspended in a minimal volume of dilute buffer, and dialyzed overnight against two 1-liter volumes of a buffer containing 20 mM sodium-potassium phosphate (pH 6.9), 1 mM EDTA-(Na), and 25 mM β-mercaptoethanol. The enzyme extract was lyophilized to yield 37 mg white powder. It was stored at −15 C over a desiccant.

Estimation of Enzyme Activity. A typical reaction mixture contained 1 × 10⁻³ μmol GDP-d-mannose labeled with ¹⁴C in the d-mannosyl moiety (about 0.01 μCi), 0.15 μmol NADPH, 15 μl enzyme (1 mg lyophilized [NH₄]₂SO₄ fraction/10 μl water), 3 μmol Tris-HCl buffer (pH 8) in a total volume of 25 μl. The mixture was sealed in a thin-walled (1.2-mm) glass capillary and incubated for 45 min at 37 C. The contents were mixed with 0.05 μmol GDP-D-glucose and applied directly to a paper moistened with ammonium formate buffer (pH 2.7) on the electrophoresis apparatus. After electrophoresis, the paper was dried in the hood, and GDP-hexoses were located by the UV-absorption of the standard GDP-D-glucose. They were eluted from the paper with water and evaporated to dryness in vacuo on a plastic planchette. The residue was taken up in 10 μl 1 M trifluoroacetic acid containing 0.2 μmol each L-fucose and D-mannose and heated in a sealed capillary at 100 C for 10 min. The hydrolysate was chromatographed in Solvent I with 0.2 μmol each L-fucose and D-mannose applied near the edges of the paper as standards. The standard sugars were made visible with the p-anisidine-HCl reagent (10) and used to locate the labeled sugars. Those portions of the paper were cut out, and radioactivity in the compounds was estimated as described above. (Occasionally, to check the overall process, ¹⁴C-labeled compounds were located by exposure of the papers to x-ray film.)

To measure the formation of the intermediate, GDP-4-keto-6-deoxy-d-mannose (9), a similar enzyme reaction mixture was prepared except that NADPH was omitted. After incubation, 0.2 μmol GDP-d-glucose and 2 μmol freshly dissolved NaBH₄ were added to the mixture on a planchette, and the mixture was allowed to react for 30 min in a moist atmosphere. The mixture then was electrophoresed, hydrolyzed, and chromatographed as before. In this case, the standards l-rhamnose and 6-deoxy-l-talose were used to locate the expected products of reduction of the 4-keto intermediate, d-rhamnose and 6-deoxy-d-talose. (6-Deoxy-l-talose was made by the molybdate-catalyzed epimerization of l-fucose which produces a mixture of l-fucose (80%) and 6-deoxy-l-talose (20%) [44].)

Incorporation of Tritium from [³H]H₂O into the Products of the Fucose-synthesizing System. GDP-d-Mannose, 5 × 10⁻³ μmol, 0.1 μmol NADPH, and 1.5 μmol Tris-HCl (pH 8) were dried in vacuo in a small plastic reaction tube. To the residue was added 1 mg lyophilized flax enzyme extract and 12 μl [³H]H₂O (90 μCi/μmol). The stoppered tube was incubated for 60 min at 37 C. The reaction was stopped by the addition of 0.1 ml 95% ethanol. Ethanol was removed in vacuo and 0.1 ml H₂O was added and similarly removed in vacuo over silica gel in a desiccator. The residue remaining was mixed with 0.05 μmol carrier GDP-D-glucose in 20 μl H₂O and subjected to electrophoresis on paper at pH 2.7. The sugar nucleotides subsequently eluted from the electrophoresis paper were separated by chromatography in Solvent II for 48 h at room temperature. Residual ammonium acetate was removed from the chromatogram by irrigating the paper in the tank overnight with 95% ethanol. Authentic GDP-d-mannose and GDP-l-fucose were usually mixed with the sample and were located by their UV absorption.

RESULTS AND DISCUSSION

The enzymic synthesis of GDP-l-fucose has been shown to include the formation of a 4-keto intermediate (9, 11, 12). The other steps in its synthesis are thought to be analogous to those established for dTDP-l-rhamnose by Melo et al. (13, 14) and Gabriel and Lindquist (8) in a definitive series of experiments with deuterium- and tritium-labeled compounds. That pathway if applied to the synthesis of GDP-l-fucose, would be as shown in Figure 1. There are two or more enzymes involved in the overall process. The first catalyzes the oxidation at carbon 4 and the reduction at carbon 6. In the case of the dTDP-D-glucose oxidoreductase, NAD firmly bound to the enzyme serves as the carrier of the hydride ion. The second enzyme (or enzymes) catalyzes the epimerizations at carbons 3 and 5 and the stereospecific reduction of the 4-keto group by exogenous NADPH. Exchange of H with the medium occurs during the formation and reduction of enediol intermediates at those positions where epimerization occurs and at carbon 5 upon reduction of the 4-keto-5,6-glucosone derivative (6).

If that scheme were followed, one would expect an exchange of a proton with H₂O at carbon 5 of the mannose in GDP-4-keto-6-deoxy-d-mannose (13) and further exchanges at carbons 3 and 5 upon epimerization through the formation of enediol intermediates (14). The 4-keto-6-deoxy-d-mannose intermediate, unlike 4-keto-6-deoxy-d-glucose, is extremely labile and virtually impossible to isolate in that form.

In Table 1, the results of an experiment using a substrate differentially labeled with tritium are presented. Reactions 1 and 3 are control mixtures incubated with BSA in place of enzyme to measure the actual recovery of radioactivity by the methods outlined above. Reaction 2 demonstrates that essentially all the tritium present in GDP-d-mannose is lost from that molecule in the course of the reaction and does not appear in GDP-l-fucose. (The tritium could be recovered from the mixture in a volatile fraction, presumably H₂O.) There is also loss of label from the GDP-l-galactose present in the sugar nucleotide preparation. Its loss is thought to be due to the action of a small amount of the GDP-d-mannose:GDP-l-galactose epimerase in the enzyme preparation (2).

Reaction 4 in which ¹⁴C-labeled GDP-d-mannose is the substrate is included to show that, under the experimental conditions, GDP-l-fucose is synthesized, and reaction 5 demonstrates that the ³H-labeled GDP-d-mannose/l-galactose preparation itself does not inhibit that synthesis.

As expected, GDP-4-keto-6-deoxy-d-mannose is produced in the course of the synthesis. That is demonstrated in reaction 7 by the reduction of its hexose moieties to the two 6-deoxy-mannose 4-epimers d-rhamnose and 6-deoxy-d-talose. In reaction 6 where the tritium-labeled substrate is used, some label is retained in the 6-deoxyhexose products. This is also to be expected since, according to the scheme given in Figure 1, only the hydrogen at carbon 5 of d-mannose would be exchanged with H₂O during formation of the keto intermediate. Little tritium is lost from GDP-l-galactose during this reaction, suggesting that there is only very slight
Table 1. Reaction of GDP-3,5-d-[3H]Mannose with an Enzyme Preparation from Flax Fruits that Catalyzes the Conversion of GDP-3,5-d-Manose to GDP-1-Manose

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>Substrate</th>
<th>Amount Recovered in Hexosyl Moiety</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G-Man</td>
<td>D-Man</td>
</tr>
<tr>
<td>1. Control (bovine serum albumin substituted for enzyme)</td>
<td>GDP-3,5-d-[3H]mannose-1-galactose</td>
<td>1143</td>
</tr>
<tr>
<td>2. Flax enzyme extract and NADPH</td>
<td>GDP-3,5-d-[3H]mannose-1-galactose</td>
<td>329</td>
</tr>
<tr>
<td>3. Control (bovine serum albumin substituted for enzyme)</td>
<td>GDP-d-[14C]mannose-1-galactose</td>
<td>129</td>
</tr>
<tr>
<td>5. Flax enzyme extract and NADPH</td>
<td>GDP-d-[14C]mannose and GDP-3,5-d-[3H]mannose-1-galactose</td>
<td>284</td>
</tr>
<tr>
<td>6. Flax enzyme without NADPH; mixture reduced with NaBH₄</td>
<td>GDP-3,5-d-[3H]mannose-1-galactose</td>
<td>789</td>
</tr>
<tr>
<td>7. Flax enzyme without NADPH, mixture reduced with NaBH₄</td>
<td>GDP-d-[14C]mannose</td>
<td>728</td>
</tr>
</tbody>
</table>

epimerase activity in the enzyme preparation under the conditions of these experiments. By demonstrating that tritium on carbons 3 and 5 of D-mannose in the sugar nucleotide is exchanged with protons in the medium during the synthesis of GDP-1-fucose, it seemed desirable to demonstrate the reverse of that process, that is, the incorporation of tritium at those positions from [3H]H₂O in the medium. To that end, enzyme reaction mixtures were incubated with [3H]H₂O, and GDP-1-fucose was isolated as described under “Materials and Methods.” Thus, a reaction mixture containing 12 µl [3H]H₂O yielded 67,000 cpm or about 0.1 µCi 3H-labeled GDP-1-fucose/5 × 10⁻⁴ µmol GDP-1-fucose (2 µCi/µmol). (Upon isolation of fucose from that compound by hydrolysis and chromatography, it was found that all the 3H label in the sugar nucleotide was in the 1-fucose moiety.) If two protons are exchanged with the protons of [3H]H₂O/molecule GDP-1-fucose formed, the maximum possible specific radioactivity of the product would be 90 µCi/µmol. Thus, 3%, or about 2%, of that incorporation was realized.

To determine the location of tritium in 1-fucose, oxidation of the sugar nucleotide and the free sugar with sodium periodate was carried out under a variety of conditions. If 1-fucose was labeled at carbons 3 and 5 as expected, upon periodate oxidation tritium should be found in formic acid from carbon 3 and in acetaldehyde from carbon 5.

When 3H-labeled GDP-1-fucose was treated for various times ranging from 2 to 15 h (pH 5.3) in 25 mM sodium periodate and formic acid was isolated by microdistillation from the frozen state, about one-third the total 3H was recovered in the distillate ([3H]formic acid from carbon 3 of 1-fucose) (3). Similarly when 1-[3H]fucose obtained from the sugar nucleotide by acid hydrolysis was oxidized with periodate and the [3H]acetaldheyde produced was collected as the dienonan derivative after Gabriel and Ashwell (7), a portion of the label was found in the acetaldheyde-dimen complex, but the results of repeated experiments were erratic. Furthermore, observations on the periodate oxidation of suppos-edly authentic 1-[6-3H]fucose (synthesized chemically by the Amersham Corp.) suggested that the tritium at carbon 6 exchanged with protons in the medium during the oxidation at pH 5.3 or at pH 2.

Because of the difficulties experienced with the periodate oxidations, I concluded that this information could not be used to fix the position of label in the molecule. There is no doubt that 3H is enzymatically incorporated into GDP-1-fucose from [3H]H₂O and that some of it is at carbon 3 of 1-fucose and some is at carbon 5 (or 6), but the data cannot be interpreted further than that. In addition to the apparent labilization of tritium on the sugar by periodate oxidation, it is possible that this rather crude enzyme fraction catalyzed unrelated reactions in which 3H-labeled NADPH was produced. That might explain the unequal distribution of tritium between carbon 3 and the remainder of the molecule since the hydride ion from NADPH is presumed to be introduced at carbon 4.

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