Nucleoside Diphosphate-sugar 4-Epimerases

I. Uridine Diphosphate Glucose 4-Epimerase of Wheat Germ

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Abstract. Uridine diphosphate (UDP)-glucose 4-epimerase (EC 5.1.3.2) has been purified over 1000-fold from extracts of wheat germ by MnCI2 treatment, (NH4)2SO4 fractionation, Sephadex column chromatography, and adsorption onto and elution from calcium phosphate gel. The enzyme has a pH optimum of 9.0. Km values are 0.1 mM for UDP-D-galactose and 0.2 mM for UDP-D-glucose. NAD is required for activity; Kd = 0.04 mM. NADH is an inhibitor strictly competitive with NAD; Kd = 2 μM. Wheat germ also contains UDP-L-arabinose 4-epimerase (EC 5.1.3.5) and thymidine diphosphate (TDP)-glucose 4-epimerase which are distinct from UDP-glucose 4-epimerase.

Materials and Methods

Raw wheat germ was obtained from Walnut Acres, Penn's Creek, Pennsylvania. Sephadex G-75, G-100, and G-200 were purchased from Pharmacia Corporation. D-glucose, D-galactose, NAD, NADH, UDP-D-glucose, UDP-D-galactose, and UDP-D-xylose were purchased from Sigma Chemical Company. UDP-D-glucose and TDP-D-glucose labeled with 14C in the D-glucosyl moiety were purchased from Calbiochem. UDP-D-xylose labeled with 14C in the D-xylosyl moiety was prepared according to Ankell and Feingold (1). Enzyme grade ammonium sulfate was a product of Mann Research Laboratory. Calcium phosphate gel was obtained from Nutritional Biochemical Corporation. Glucostat was purchased from Worthington Biochemical Corporation. All other chemicals, unless otherwise noted, were of reagent grade and were obtained from commercial sources.

D-Galactose dehydrogenase (EC 1.1.1.48) and L-arabinose dehydrogenase (EC 1.1.1.46) were prepared from Pseudomonas saccharophila (3). UDP-glucose dehydrogenase (EC 1.1.1.22), specific activity about 3 units per min per mg protein, was prepared from beef liver (17).

Two buffer solutions were used in the enzyme purification: A. 0.1 M sodium and potassium phosphate, pH 7.0. B. 0.01 M sodium and potassium phosphate, pH 7.0. Both A and B contained 0.5 g of EDTA and 0.5 ml of 2-mercaptoethanol per liter of solution.

Protein was determined by the Lowry method (9) with bovine serum albumin as standard. UDP-glucose 4-epimerase was assayed either by a 1- or 2-step method.

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**One-step Assay.** This assay was performed essentially as described by Maxwell (10). 0.5 ml cuvettes contained (µmoles): NAD, 0.5; glycine buffer, pH 9.0, 60; 1 unit of UDP-glucose dehydrogenase, and UDP-glucose 4-epimerase in a total volume of 0.4 ml. The reaction was started by addition of 5 µl of 0.01 M UDP-glucose; the absorbancy increase at 30º was followed continuously at 340 mp in a Beckman Spectrophotometer equipped with the Gilford Multiple Sample Recorder. A control was run without substrate to compensate for unspecified reduction of NAD. A unit of enzyme is defined as the amount of enzyme required to convert 1 µmole of UDP-glucose to UDP-glucose per min under the conditions of assay.

**Two-step Assay.** (This method also was used for assay of TDP-glucose 4-epimerase). The reaction mixture contained enzyme, substrate and NAD in glycine buffer (pH 9.0). After incubation for 2.5 min at 30º, the reaction mixtures were held at 100º for 1 min, an excess of UDP-glucose dehydrogenase and NAD was added, and the total absorbancy increase at 340 mp was noted. Under these conditions, the reaction was linear with time for at least 6 min. Since UDP-glucose dehydrogenase is greatly inhibited by low concentrations of NADH (17), it was often impossible to use this enzyme in assay procedures. In these cases, as well as when UDP-glucose was used as substrate, the concentration of UDP-glucose or UDP-glucose was determined in the reaction mixture was ascertained by analyzing for the free sugar released from the sugar nucleotide by acid hydrolysis. The assay mixtures were made 0.1 N in HCl, heated at 100º for 15 min, neutralized, and the glucose or UDP-glucose in the hydrolysate was determined with Glucostat or UDP-glucose dehydrogenase, respectively.

**Assay of UDP-1-arabinose 4-Epimerase (EC 5.1.3.5).** Reaction mixtures contained (µmoles): glycine buffer, pH 9.0, 50; UDP-β-xylene, 4.0; NAD (where indicated), 0.1; and enzyme preparation, 0.1 ml; in a total volume of 0.35 ml. At 40 min at 30º, the reaction was stopped by heating at 100º for 1 min. Precipitated protein was removed by centrifugation, the supernatant fluid was made 0.1 N in HCl, heated for 15 min at 100º, cooled to room temperature, and the pH was adjusted to 8.5 with 7.5 N NH₄OH. 1-arabinose present in the hydrolysate was determined as follows: Reaction mixtures (0.45 ml) contained (µmoles) NAD, 0.1; tris-NaOH buffer, pH 8.5, 60; and from 0.1 to 0.2 ml of hydrolysate; 1-arabinose dehydrogenase (1 unit) was added to start the reaction and the total absorbancy increase at 340 mp was measured. The specific activity is defined as for UDP-glucose 4-epimerase.

**Purification of UDP-Glucose 4-Epimerase.** Except where otherwise indicated, the following procedures were performed at 0 to 4º. Centrifugations were carried out at 10,000g for 20 min.

**Preparations of Crude Extract.** 250 g of raw wheat germ was stirred gently with 1200 ml of buffer A for 1 hr. The thick slurry was centrifuged, yielding about 1 liter of turbid supernatant fluid (crude extract).

**MnCl₂ Treatment.** Under efficient stirring, 0.5 M MnCl₂ was added to the crude extract (1030 ml) to a final concentration of 0.015 M; after 5 min, the mixture was centrifuged and the supernatant fluid was retained (MnCl₂ supernatant).

**NH₄₂SO₄ Fractionation.** To the MnCl₂ supernatant (1025 ml), solid ammonium sulfate was added to 20% saturation and the precipitate was discarded. The supernatant solution was then brought to 40% saturation; the resultant precipitate was collected by centrifugation and dissolved in buffer A to a final volume of 140 ml ((NH₄)₂SO₄ fraction).

**Sephadex Column Chromatography.** The brownish (NH₄)₂SO₄ fraction was placed on a 5.5 cm diameter column containing 1900 ml of Sephadex G-75 equilibrated with buffer B. The protein was eluted with buffer B; 20 ml fractions were collected. Active fractions were pooled, solid ammonium sulfate was added to 25% saturation, and the precipitate was removed by centrifugation. Solid ammonium sulfate was added to the supernatant solution to 40% saturation and the precipitate was dissolved in buffer A, to a final volume of 80 ml [Sephadex G-75 (I)]. The solution was re-chromatographed on a 4 cm diameter column containing 1000 ml of Sephadex G-75 equilibrated with buffer B. The same buffer was used as eluent and 10 ml fractions were collected. Active fractions were pooled and fractionated with ammonium sulfate as above, and the precipitated protein was dissolved in buffer A to a final volume of 35.5 ml [Sephadex G-75 (II)]. The solution was re-chromatographed on a 950 ml Sephadex G-200 column (4 cm diameter) as described. 7.5 ml fractions were collected; active fractions were pooled and fractionated with ammonium sulfate between 30 and 40% saturation. The precipitated protein was dissolved in a small volume of buffer A to yield 4.4 ml of solution (Sephadex G-100). The so-obtained enzyme fraction again was chromatographed on a 600 ml Sephadex G-200 (3 cm diameter) column using buffer B as eluent; 5 ml fractions were collected. Active fractions were pooled and brought to 40% saturation with solid ammonium sulfate and the precipitate was dissolved in a small volume of buffer A to yield 3.4 ml of solution (Sephadex G-200).

**Calcium Phosphate Gel Adsorption.** The Sephadex G-200 fraction was diluted with buffer B to give a protein concentration of about 1 mg/ml. Calcium phosphate gel (1.5 mg dry wt/mg protein) then was added with stirring and the suspension was stirred occasionally for 20 min. The suspension then was centrifuged at 1500g for 5 min, the supernatant fluid was discarded, and the gel was washed 4 times by suspension in 50 ml buffer B and recentrifugation, followed by a single washing with 0.02 M phosphate buffer, pH 7.0. The enzyme was eluted from the gel by suspension in 5 ml of 0.1 M phosphate buffer,
pH 8.0, for 10 min. After recovery of the gel by centrifugation, the procedure was repeated once with 5 more ml of buffer. The eluates were combined and brought to 80% saturation with solid ammonium sulfate; the precipitated protein was dissolved in 2.5 ml of 0.2 M glycine buffer, pH 9.0 (calcium phosphate gel eluate). This enzyme preparation was used for most of the subsequent experiments.

**Results**

**Enzyme Purification.** The enzyme purification is summarized in Table I. Over 1000-fold purification was achieved, with recovery of about 10% of the initial activity. The final specific activity of the enzyme was 0.75, which is slightly higher than the specific activity reported for the enzyme from calf liver (10) but much lower than that of E. coli (16) or yeast enzyme (4).

**Reaction Products and Equilibrium Constant.** UDP-β-glucose labeled with 14C in the β-glucosyl moiety was incubated for 12 hr with calcium phosphate gel eluate in the standard 2-step assay, reaction mixture except that the final volume was 50 µl. At the end of this time, the presence of both 14C-UDP-β-glucose and 14C-UDP-β-galactose in the mixture was demonstrated as described by Feingold et al. (6). The equilibrium constant K for the reaction UDP-β-galactose ↔ UDP-β-glucose, determined from either direction with the 2-step assay, was 3.1. This agrees with previously reported values for the yeast enzyme (7), but is slightly lower than the value reported for the epimerase from E. coli, 3.5 (16). The presence of UDP-L-arabinose 4-epimerase or UDP-glucose 4-epimerase in enzyme preparations was demonstrated in the same way, using UDP-β-xylene or UDP-glucose labeled with 14C in the glycosyl moiety.

**pH Optimum.** UDP-Glucose 4-epimerase has a sharp pH optimum at 9.0 (Fig. 1).

**Relationship Between Substrate Concentration and Reaction Rate.** The effect of substrate concentration on the reaction rate starting from either UDP-β-galactose or UDP-β-glucose is shown in Fig. 1. The effect of pH on the activity of UDP-glucose 4-epimerase. The reaction mixture contained 0.2 µ mole UDP-β-galactose, 0.1 µ mole NAD and 0.012 unit of UDP-glucose 4-epimerase in a total volume of 0.35 ml of buffer of the indicated pH. Buffers used were: between pH 4 and 5, 0.2 M sodium acetate; between pH 7 and 8, 0.2 M sodium and potassium phosphate; between pH 9 and 11, 0.2 M glycine-NaOH. The 2-step assay procedure described in the text was used with the exception that the pH of the initial reaction mixture was brought to 8.5 before addition of indicator enzyme.

![Fig. 1. The effect of pH on the activity of UDP-glucose 4-epimerase.](image)

**Fig. 2. Km values calculated by the method of Lineweaver and Burk (8) are 0.1 mM for UDP-β-galactose and 0.2 mM for UDP-β-glucose.**

**Stability.** All UDP-glucose 4-epimerase activity is lost when solutions of the enzyme are stored frozen overnight; however, no activity is lost for up to about 1 month when it is stored frozen at −20° in the presence of 20% glycerol. Solutions (without glycerol) of pH ranging from 7 to 11 retain up to 40% their initial activity for a week at 0 to 4°. At lower pH values, activity is rapidly lost. 1.0 mM

**Table I. Purification of UDP-Glucose 4-Epimerase From Wheat Germ**

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Volume (ml)</th>
<th>Total activity (units)</th>
<th>Total protein (mg)</th>
<th>Specific activity (units/mg) x 10^5</th>
<th>Recovery (%)</th>
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</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1030</td>
<td>20.4</td>
<td>39,200</td>
<td>0.5</td>
<td>100</td>
</tr>
<tr>
<td>MnCl₂ supernatant</td>
<td>1025</td>
<td>20.2</td>
<td>32,000</td>
<td>0.6</td>
<td>100</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction</td>
<td>140</td>
<td>23.8</td>
<td>7,000</td>
<td>3.4</td>
<td>116</td>
</tr>
<tr>
<td>Sephadex G-75 (1)</td>
<td>80</td>
<td>17.0</td>
<td>3,550</td>
<td>4.8</td>
<td>84</td>
</tr>
<tr>
<td>Sephadex G-75 (11)</td>
<td>35.5</td>
<td>13.9</td>
<td>1,070</td>
<td>13.0</td>
<td>68</td>
</tr>
<tr>
<td>Sephadex G-100</td>
<td>4.4</td>
<td>10.8</td>
<td>173</td>
<td>62.0</td>
<td>53</td>
</tr>
<tr>
<td>Sephadex G-200</td>
<td>3.4</td>
<td>5.6</td>
<td>68</td>
<td>82.0</td>
<td>27</td>
</tr>
<tr>
<td>Calcium phosphate gel eluate</td>
<td>2.5</td>
<td>2.4</td>
<td>3.2</td>
<td>750.0</td>
<td>12</td>
</tr>
</tbody>
</table>
cysteine, dithiothreitol or 2-mercaptoethanol cause rapid inactivation of purified enzyme, although they do not inhibit less purified fractions.

NAD Requirement. Ammonium sulfate fraction (1 ml) in buffer A containing 50 mg of protein was treated with 50 mg of acid-washed Norit A. After 30 min at 4°C, the charcoal was removed by centrifugation and the activity of the enzyme was assayed by the 2-step method in the presence of added NAD (0.25 mM) as well as in its absence. In the latter case the initial incubation was for 10 min. Untreated enzyme also was assayed with and without NAD. As can be noted in Table II, UDP-glucose 4-epimerase activity is stimulated 14-fold upon addition of NAD to the 20 to 40% (NH₄)₂SO₄ fraction. All activity is abolished upon treatment with charcoal, while NAD restores to the charcoal-treated fraction 80% of the activity obtained by addition of NAD to the untreated enzyme. The enzyme was not reactivated, however, by 0.25 mM NAD which had been pretreated with NADase (EC 3.2.2.5). That the endogenous activator is NAD was shown by inactivation of the enzyme by treatment with NADase. 20 to 40% (NH₄)₂SO₄ fraction (30 mg in 0.5 ml of buffer A) was incubated with 200 μg of NADase for 10 min at 20°C and the activity of the mixtures then was determined by the 2-step assay, using a 10 min incubation period. A control was run without NADase treatment. No activity could be detected in the NADase-treated enzyme, while the untreated control retained its initial activity. The NAD requirement appears to be specific, since neither NADP nor the NAD analogs (0.25 mM); α-NAD, ethylthioctic acid, ethylpyridylketone and thionicotinic acid could substitute for NAD in activating the enzyme (calcium phosphate gel eluate). Kinetic studies of the activation of UDP-glucose 4-epimerase by NAD and its inhibition by NADH are presented in Fig. 3. Kₐ for NAD calculated by the method of

Table II. Effect of NAD on the Activity of UDP-Glucose 4-Epimerase

The reaction mixture contained 0.2 μmole UDP-β-galactose, 0.1 μmole NAD and 10 μl of 20 to 40% (NH₄)₂SO₄ fraction or 100 μl of 40 to 60% (NH₄)₂SO₄ fraction in a total volume of 0.35 ml in 0.2 M glycine buffer, pH 9.0. The 2-step assay procedure described in the text was used.

<table>
<thead>
<tr>
<th>Enzyme fraction</th>
<th>Specific activity × 10⁴ Additions</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>NAD</td>
</tr>
<tr>
<td>20–40% (NH₄)₂SO₄ fraction Control</td>
<td>2.3</td>
</tr>
<tr>
<td>Charcoal-treated</td>
<td>0</td>
</tr>
<tr>
<td>40–60% (NH₄)₂SO₄ fraction Control</td>
<td>0</td>
</tr>
<tr>
<td>Charcoal-treated</td>
<td>0</td>
</tr>
</tbody>
</table>

Lineweaver and Burk is 0.04 mmol; NADH is an inhibitor strictly competitive with NAD: Kᵢ = 2 μmol.

Presence of UDP-1-Arabinoæ and TDP-Glucose 4-Epimerases. Calcium phosphate gel eluate catalyzes the interconversion of UDP-β-xylose and UDP-1-
arabinose upon prolonged incubation; however, the UDP-l-arabinose 4-epimerase activity of this fraction is negligible when assayed as described under Methods. Accordingly, less purified fractions were examined in order to compare the properties and distribution of the UDP-glucose 4-epimerase and UDP-l-arabinose 4-epimerase in wheat germ extracts. The MnCl<sub>2</sub> supernatant was fractionated as described between 20 and 40% (NH₄)₂SO₄ saturation; the resultant supernatant fluid was not discarded as in the usual purification scheme but was further fractionated between 40 and 60% (NH₄)₂SO₄ saturation. The fractions were each dissolved in an equal volume of buffer A and further investigated. When the fraction which precipitated between 20 to 40% (NH₄)₂SO₄ saturation was tested for UDP-l-arabinose 4-epimerase, none could be detected with the standard assay. However, when the initial incubation was prolonged for 18 hr, activity could be detected in the 20 to 40% (NH₄)₂SO₄ fraction, corresponding roughly to a specific activity of 2 × 10⁴. The protein fraction which precipitated at 40 to 60% (NH₄)₂SO₄ saturation contained less than 0.1% of UDP-glucose 4-epimerase activity as the 20 to 40% (NH₄)₂SO₄ fraction; on the other hand, the UDP-l-arabinose 4-epimerase activity of the 40 to 60% (NH₄)₂SO₄ fraction was relatively high. The ratio of specific (total) activity with UDP-D-glucose to that with UDP-D-xylene was approximately 150 in the 20 to 40% (NH₄)₂SO₄ fraction, while it was 1 in the 40 to 60% (NH₄)₂SO₄ fraction. Furthermore, UDP-glucose 4-epimerase activity in both fractions was affected identically by NAD and charcoal treatment, which had no effect on UDP-l-arabinose 4-epimerase activity.

When the 20 to 40% (NH₄)₂SO₄ fraction was examined with either the 2-step assay or by use of ¹⁴C-labeled TDP-D-glucose in the presence of NAD, no TDP-glucose 4-epimerase activity could be detected. However, the 40 to 60% (NH₄)₂SO₄ fraction had appreciable activity, equivalent to a specific activity of 6 × 10⁴ as determined by the 2-step assay with an initial incubation period of 5 min at 30°. That this activity was due to TDP-glucose 4-epimerase was shown by the presence of ¹⁴C-labeled galactose in addition to ¹⁴C-labeled glucose in a hydrolyzate of a reaction mixture which initially contained ¹⁴C-labeled TDP-D-glucose and 40 to 60% (NH₄)₂SO₄ fraction.

**Discussion**

The UDP-glucose 4-epimerase of wheat germ is similar to that of mammalian tissues in its requirement for NAD and its inhibition by NADH. The only notable difference in kinetic properties is in Kₘ for NAD; 40 μM for wheat germ enzyme as opposed to 0.2 μM for the liver 4-epimerase. The enzyme is excluded from Sephadex G-75 and emerges slightly after the solvent front on columns of Sephadex G-100. Since the upper limit of the fractionation range of Sephadex G-100 is 1.5 × 10⁶ and that of Sephadex G-75 is 0.7 × 10⁶, the molecular weight of UDP-glucose 4-epimerase is in the order of 10⁶ daltons.

While in *E. coli* the 4-epimerization of UDP-D-glucose and UDP-l-arabinose is catalyzed by the same enzyme (2), in *Saccharomyces fragilis* separate enzyme activities for each substrate are present (15). In wheat germ also the reactions are catalyzed by different enzymes. This is shown by: A) the distribution of enzyme activity in ammonium sulfate fractions, the ratio of UDP-glucose to UDP-l-arabinose 4-epimerase being 150 in the 20 to 40% (NH₄)₂SO₄ fraction and 1 in the 40 to 60% (NH₄)₂SO₄ fraction; and B) the different effect of NAD and charcoal on the separate activities.

In addition to UDP-glucose and UDP-l-arabinose 4-epimerases, wheat germ contains low but detectable TDP-glucose 4-epimerase. Neufeld reported that partially purified TDP-glucose 4-epimerase from germinating seeds of *Phaseolus aureus* catalyzed the 4-epimerization of UDP-D-glucose as well as of TDP-D-glucose (12). The results presented in this paper show that in wheat germ, UDP-glucose 4-epimerase is distinct from TDP-glucose 4-epimerase, since the 20 to 40% (NH₄)₂SO₄ fraction, which has high UDP-glucose 4-epimerase activity, is completely lacking in TDP-glucose 4-epimerase, while the 40 to 60% (NH₄)₂SO₄ fraction, low in UDP-glucose 4-epimerase activity, contains appreciable TDP-glucose 4-epimerase. The data are less conclusive in regard to whether the same protein fraction can catalyze the 4-epimerization of UDP-l-arabinose and TDP-D-glucose. The 20 to 40% (NH₄)₂SO₄ fraction, which contains some UDP-l-arabinose 4-epimerase activity, has no detectable TDP-glucose 4-epimerase activity, even when tested with radioactive substrate: both activities, however, are present in the 40 to 60% (NH₄)₂SO₄ fraction. Were TDP-D-glucose and UDP-l-arabinose epimerized by the same enzyme, one would expect to find both activities in fractions containing UDP-l-arabinose 4-epimerase; thus our results suggest that these 2 activities likewise are attributable to separate enzymes.

Since UDP-glucose 4-epimerase requires NAD and is inhibited by NADH, the ratio of NADH/NAD could play a role in controlling the conversion of UDP-D-glucose to UDP-D-galactose in higher plants, increasing the activity of the enzyme when this ratio is low, and decreasing it when it is high. A similar effect of the NADH/NAD ratio of UDP-glucose 4-epimerase in L-cells has been suggested by Robinson et al. (14). UDP-l-arabinose 4-epimerase, on the other hand, is not under this control, since its activity is not enhanced by NAD. If this 4-epimerase is similar to UDP-glucose 4-epimerase in its reaction mechanism, it probably has tightly bound NAD, like the UDP-glucose 4-epimerase of *Saccharomyces fragilis* (4,11,16). This aspect is at present under investigation.
Acknowledgment

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Literature Cited