Nucleoside Diphosphate-Sugar 4-Epimerases

II. URIDINE DIPHOSPHATE ARABINOSE 4-EPIMERASE OF WHEAT GERM

DER-FONG FAN* AND DAVID SIDNEY FEINGOLD*

Department of Microbiology, University of Pittsburgh, School of Medicine, Pittsburgh, Pennsylvania 15213

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ABSTRACT

Uridine diphosphate (UDP)-arabinose 4-epimerase (EC 5.1.3.5) has been purified to at least 20-fold from wheat germ by MnCl2 treatment, (NH4)2SO4 fractionation, dialysis, and Sephadex and diethylaminoethyl cellulose column chromatography. The enzyme has no action on UDP-d-glucose, UDP-d-glucuronic acid, or TDP-d-glucose. The pH optimum is 8.0. Km values are 1.5 mM for UDP-d-xylene and 6.5 mM for UDP-L-arabinose. The equilibrium constant, K, for the reaction UDP-L-arabinose = UDP-d-xylene is 1.25. The enzyme is neither activated by nicotinamide adenine dinucleotide nor inhibited by reduced nicotinamide adenine dinucleotide. It is completely inhibited by p-chloromercuriphenylsulfonate; the inhibition is reversed by cysteine.

Crude preparations from higher plants catalyze the 4-epimerization of UDP-d-glucose, TDP-d-glucose, UDP-d-xylene, and UDP-d-glucuronic acid (6, 10, 11). In a previous publication we suggested that wheat germ contains an enzyme which specifically catalyzes the interconversion of UDP-d-xylene and UDP-L-arabinose (5). The partial purification and some properties of this enzyme, UDP-arabinose 4-epimerase, are described in this communication.

MATERIALS AND METHODS

Raw wheat germ was obtained from Walnut Acres, Penn's Creek, Pennsylvania. UDP-U-14C-d-glucose, UDP-U-14C-d-glucuronic acid, UDP-U-14C-d-xylene, and UDP-U-14C-L-arabinose were purchased from New England Nuclear Corp. Unlabeled UDP-L-arabinose was prepared as described in "Results." All other chemicals used were of reagent grade and were obtained commercially. Two buffer solutions were used: buffer A: 0.1 M sodium phosphate, pH 7.0; buffer B: 0.01 M sodium phosphate, pH 7.0. Both buffers A and B contained 0.5 g of EDTA and 0.5 ml of 2-mercaptoethanol per liter.

Whatman 3MM paper washed with 1% oxalic acid was used for paper chromatography and paper electrophoresis. Descending paper chromatography was carried out in the following solvent systems (v/v): solvent 1: 95% ethanol-1 M ammonium acetate, pH 7.5 (7:3); solvent 2: n-butanol-pyridine-water (10:3:3);

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4 Abbreviations: GLC: gas-liquid chromatography; TMS: trimethylsilyl; DEAE: diethylaminoethyl; PCMP: p-chloromercuriphenylsulfonate.
with occasional shaking, 0.5 \mu l of the mixture was chromatographed isothermally at 150 C. The area of peaks with the same retention time as standard TMS-D-xylene and TMS-L-arabinose was determined, and the quantity of L-arabinose in the reaction mixture was calculated from the ratio of these areas. Under these conditions, D-xylene and L-arabinose can be clearly differentiated; the area obtained from L-arabinose is 1.05 that obtained from an equimolar quantity of D-xylene. A unit of enzyme is defined as the amount of enzyme required to convert 1 amole of UDP-D-xylene to UDP-L-arabinose per min under the conditions of assay. In some experiments an assay based on the determination of radioactive L-arabinose in hydrolysates of reaction mixtures was used. Reaction mixtures, containing labeled UDP-D-xylene (specific radioactivity 172.5 curie/mole) and 40 \mu l of enzyme solution, were incubated in a capillary tube at 30 C for 20 min. The contents of the tube were made 0.1 N in HCl, the sugar nucleotides were hydrolyzed for 15 min at 100 C, and the hydrolysate was taken to dryness as described above. In order to avoid interference by salt during the subsequent paper chromatography, the sugars were extracted from the dry residue with pyridine and applied to the paper, which was then developed in solvent 2. The radioactive spots of the separated D-xylene and L-arabinose were determined with the strip scanner.

**Purification of UDP-Arabinose 4-Epimerase.** All purification procedures were carried out at 0 to 4 C. Centrifugations were performed at 10,000 g for 20 min.

**Preparation of Crude Extract.** Raw wheat germ, 2 kg, was stirred for 1.5 hr with 13 liters of buffer A. The thick slurry was squeezed through four layers of cheesecloth to yield approximately 10 liters of turbid supernatant fluid (crude extract).

**MnCl\(_2\) Treatment.** Under vigorous stirring, 0.5 M MnCl\(_2\) was added to the crude extract to a final concentration of 0.015 M. After 5 min, the mixture was centrifuged and the supernatant fluid was retained (MnCl\(_2\) supernatant). UDP-arabinose 4-epimerase could not be assayed accurately by GLC in either crude extract or MnCl\(_2\) supernatant, because of the high endogenous L-arabinose content and low specific activity of these fractions. However, enzymatic activity could be demonstrated with labeled UDP-D-xylene as described above.

**\(\text{(NH}_4\text{)}_2\text{SO}_4\) Fractionation.** To the MnCl\(_2\) supernatant (10 liters), solid ammonium sulfate was added to 50\% saturation, and the precipitated protein was discarded. The supernatant fluid was then brought to 65\% saturation; the resultant precipitate was collected by centrifugation and dissolved in buffer A to a final volume of 250 ml (\(\text{(NH}_4\text{)}_2\text{SO}_4\) fraction).

**Dialysis.** The \(\text{(NH}_4\text{)}_2\text{SO}_4\) fraction was dialyzed for 18 hr against 19 liters of buffer A; the retentate was diluted with buffer A to 800 ml and fractionated with ammonium sulfate. Protein which precipitated between 50 and 70\% \((\text{NH}_4\text{)}_2\text{SO}_4\) saturation was collected and dissolved in 120 ml of buffer A. This solution was dialyzed against 18 liters of buffer A as above, and the retentate was diluted with buffer A to 500 ml and again fractionated with solid ammonium sulfate; the fraction which precipitated between 50 and 70\% saturation was dissolved in 90 ml of buffer A (dialyzed fraction).

**Sephadex G-75 Column Chromatography.** The dialyzed fraction was placed on a 5.5-cm diameter column containing 1900 ml of Sephadex G-75 equilibrated with buffer A. The protein was eluted with buffer A; 15-ml fractions were collected, and active fractions were pooled and fractionated with solid ammonium sulfate. The protein which precipitated between 50 and 70\% \((\text{NH}_4\text{)}_2\text{SO}_4\) saturation was collected and dissolved in 50 ml of buffer A (Sephadex G-75 fraction).

**DEAE-cellulose Column Chromatography.** The Sephadex G-75 fraction was dialyzed against 10 liters of buffer B for 5 hr and then placed on a 4.5-cm diameter column containing 1500 ml of DEAE-cellulose equilibrated with buffer B. The column was eluted with buffer B, and 20-ml fractions were collected. Active fractions (600 ml) were pooled and brought to 75\% \((\text{NH}_4\text{)}_2\text{SO}_4\) saturation. After centrifugation, the protein was present as a felt floating at the top of the solution. This material was collected with a glass rod and dissolved in 24 ml of buffer A (DEAE-cellulose fraction).

**Sephadex G-100 Column Chromatography.** DEAE-Cellulose fraction was chromatographed on a Sephadex G-100 column (4 cm in diameter); 10-ml fractions were collected. Active fractions were pooled and brought to 75\% saturation with solid ammonium sulfate. The protein felt was collected as above and dissolved in 7 ml of buffer A. This enzyme preparation was used for all subsequent studies.

**RESULTS**

**Enzyme Purification.** The enzyme purification is summarized in Table I. Over 20-fold purification was obtained with recovery of 17\% of the activity present in the \((\text{NH}_4\text{)}_2\text{SO}_4\) fraction. The final specific activity was 0.01.

**Stability.** The enzyme is stable for at least 3 weeks at 0 to 4 C, but it loses 50\% of its initial activity after 2 months at this temperature or when stored frozen for 12 hr. However, \((\text{NH}_4\text{)}_2\text{SO}_4\) fraction can be stored frozen with no loss of activity. Purified enzyme loses 80\% of its initial activity when held at 50 C for 1 min.

**Fatty Acid Content.** Enzyme eluted from DEAE-cellulose or Sephadex G-100 columns was not precipitated by centrifugation after addition of ammonium sulfate but formed a felt which floated on the ammonium sulfate solution. This felt was soluble in buffer A as described; it was readily precipitated from solution 10 volumes of acetone at -20 C. The acetone-precipitated enzyme was soluble in buffer A and retained full activity. However, it could not be precipitated with ammonium sulfate but formed a felt as before. Sephadex G-100 fraction and acetone-precipitated material were examined for the presence of fatty acids as described under "Materials and Methods" and found to contain palmitic, stearic, and myristic acids in the relative quantities 8:4:1, respectively. Approximately 3\% by weight of the protein of each of these fractions consisted of fatty acids.

**Absence of Other Enzyme Activity.** The Sephadex G-100 fraction was free from UDP-glucose-, TDP-glucose-, and UDP-glucuronate-4-epimerase activity. This was established by incubating the enzyme for 2 hr with radioactive UDP-D-glucose, TDP-D-glucose, or UDP-D-glucuronic acid, hydrolyzing the sugar nucleotides at 100 C in 0.1 N HCl and examining the glycoside products by paper chromatography in solvent 2; when UDP-D-glucuronic acid was used as substrate, paper electrophoresis at pH 3.6 was used. When tested shortly after elution from Sephadex G-100, the enzyme contained a small amount of UDP-glucuronate carboxy-lyase (EC 4.1.1.35) activity. This was shown by incuba-
L-arabinose dehydrogenase.

Two ultraviolet-quenching spots were found to be present. One had the same chromatographic mobility of UDP-D-xylose, while the other corresponded in mobility to UDP-L-arabinose. The same qualitative results were obtained regardless of the starting material (UDP-D-xylose or UDP-L-arabinose). These compounds were eluted, and each was purified by chromatography in solvent 3. The so-purified compounds were subjected to paper chromatography at pH 5.0, and the relative quantities of UDP-L-arabinose and UDP-D-xylose were found to be present. One had the same chromatographic mobility of UDP-D-xylose, while the other corresponded in mobility to UDP-L-arabinose. The same qualitative results were obtained regardless of the starting material (UDP-D-xylose or UDP-L-arabinose). These compounds were eluted, and each was purified by chromatography in solvent 4. The so-purified compounds were subjected to paper chromatography at pH 3.6, and the relative quantities of UDP-L-arabinose and UDP-D-xylose were found to be present. The hydrolysis products were examined by paper electrophoresis at pH 3.6 and by paper chromatography in solvent 2. Two ultraviolet-quenching spots were present with the mobility of authentic UMP and UDP, respectively, as well as a substance which reacted with phosphohydrolase acid (2) and which had the mobility of inorganic phosphate. Two ultraviolet-quenching spots were present on the electrophoreses. Paper chromatography of the hydrolysate in solvent 2 revealed the presence of two substances with the mobility of authentic D-xylose and L-arabinose. These two sugars were also shown by GLC to be present in the hydrolysate. Glucose oxidase was used to show that the xylosyl moiety of the product obtained by 4-epimerization of UDP-L-arabinose was the D isomer (1); similarly, L-arabinose dehydrogenase was used to show that the arabinosyl moiety of the product obtained from UDP-D-xylose was the L form of the sugar (4).

The equilibrium constant, $K$, for the reaction UDP-L-arabinose $\rightleftharpoons$ UDP-D-xylose was determined starting from either direction. The reaction was performed under assay conditions, and GLC and radioactivity measurements were used to establish the relative quantities of L-arabinose and D-xylose in hydrolysates of reaction mixtures. In addition, the relative quantities of UDP-L-arabinose and UDP-D-xylose were ascertained by determination of radioactivity in the two intact sugar nucleotides after separating them by paper chromatography in solvent 3. All results agreed within experimental error. The value of $K$ for the reaction UDP-L-arabinose $\rightleftharpoons$ UDP-D-xylose is 1.25.

**pH Optimum.** UDP-arabinose 4-epimerase activity was determined using 0.2 mM sodium acetate buffer of pH 4.0 and 5.0; 0.2

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**FIG. 1.** The effect of pH on the activity of UDP-arabinose 4-epimerase. The reaction mixture contained 2 µmoles of UDP-D-xylose and 0.033 unit of UDP-arabinose 4-epimerase in a total volume of 0.40 ml of buffer of the indicated pH. Incubation was carried out at 30°C for 10 min. The UDP-arabinose 4-epimerase activity was determined by GLC assay procedure described in the text.

**FIG. 2.** Dependence of reaction rate on UDP-D-xylose concentration. The reaction mixture contained 0.025 unit of UDP-arabinose 4-epimerase and the indicated concentrations of labeled UDP-D-xylose in a total volume of 0.40 ml of 0.2 M sodium phosphate buffer, pH 8.0. Reaction time at 30°C was 15 min; the amount of UDP-L-arabinose formed was determined by measurement of radioactivity as described in the text.

**FIG. 3.** Dependence of reaction rate on UDP-L-arabinose concentration. The reaction mixture contained 0.001 unit of UDP-arabinose 4-epimerase and the indicated concentrations of labeled UDP-L-arabinose in a total volume of 65 µl of 0.2 M sodium phosphate buffer, pH 8.0. Reaction and assay conditions were identical to those described for Figure 2.
m phosphate buffer between pH 6.0 and 8.0, 0.2 m glycine-NaOH buffer of pH 9.0 and 10.0. As can be seen in Figure 1, the enzyme has an optimal pH in the vicinity of 8.0.

Dependence of Reaction Rate on Enzyme Concentration. Under conditions of assay the reaction was linear with time for at least 20 min. A linear relationship between activity and amount of protein was also obtained.

Effect of Substrate Concentration. The effect of UDP-D-xylose and UDP-L-arabinose concentration on the reaction rate is shown in Figures 2 and 3, respectively. The Km values calculated by the method of Lineweaver and Burk are 1.5 mm for UDP-D-xylose and 0.5 mm for UDP-L-arabinose.

Cofactor Requirement. NAD, NADP, NADH, NADPH, UMP, UDP, UTP, UDP-D-glucose, UDP-D-galactose, UDP-D-glucuronic acid, and TDP-D-glucose at concentrations of 0.5 mm or 2.0 mm did not affect reaction rate. Prolonged dialysis of Sephadex G-100 fractions against a large excess of buffer A or treatment of the enzyme with nicotinamide adenine dinucleotidase (EC 3.2.2.5) or charcoal did not decrease enzyme activity as compared with untreated controls.

Effect of Sulphydryl Reagents. Although 2-mercaptoethanol was used in the enzyme purification, it is not necessary for activity, since enzyme dialyzed against a large volume of buffer A was fully active. However, the enzyme was inactivated by mm p-chloromercuriphenylsulfonate. Activity was restored when cysteine (final concentration 33 mm) was added to the PCMP-inactivated enzyme. UDP-glucuronate carboxyl-lyase present in fresh Sephadex G-100 preparations was irreversibly inactivated by the PCMP treatment.

DISCUSSION

In a previous publication we suggested that wheat germ contains a specific enzyme for the 4-epimerization of UDP-L-arabinose (5). However, while UDP-arabinose 4-epimerase was clearly differentiated from UDP-glucose 4-epimerase on the basis of (a) the distribution of the separate activities among different protein fractions and (b) NAD requirement, the question of the relationship between UDP-arabinose 4-epimerase and TDP-glucose 4-epimerase was not settled. This question is resolved by the data presented in the present paper. Crude wheat germ extract catalyzes the 4-epimerization of UDP-L-arabinose, UDP-D-glucose, UDP-D-glucuronic acid, and TDP-D-glucose; the partially purified enzyme, on the other hand, only catalyzes the 4-epimerization of UDP-L-arabinose and is inert toward the other compounds. In addition, these compounds, when tested in concentrations roughly equivalent to Km for UDP-D-xylose, have no effect on the kinetic behavior of the enzyme with UDP-D-xylose.

Inactivation of the enzyme by PCMP shows its requirement for reduced thiol groups. Irreversible conformational changes do not result from such inactivation, since reactivation occurs upon cysteine treatment. Interestingly, a number of fatty acids were found to be closely associated with enzymatically active protein. From the data available it is not possible to determine whether these fatty acids, which represent about 3% by weight of the protein present, are associated with the enzyme itself or with an impurity.

NAD, either free or tightly bound, has been shown to be essential for the activity of UDP-glucose 4-epimerase (5, 8, 9, 15) and probably is required by other nucleoside diphosphate sugar 4-epimerases as well. UDP-arabinose 4-epimerase activity is unaffected by NAD, NADH, prolonged dialysis, and treatment with charcoal or nicotinamide adenine dinucleotidase. Therefore, if NAD is involved in the action mechanism of the enzyme, it must be tightly bound and not accessible to reagents such as charcoal or nicotinamide adenine dinucleotidase.

The equilibrium constant, K, for the reaction UDP-L-arabinose ⇌ UDP-D-xylose is 1.25. This figure is in reasonable agreement with the value 1.0 found for a preparation from mung beans (6), and with the value 1.1 found for the reaction catalyzed by a preparation from yeast (12). The ratio of UDP-D-glucose to UDP-D-galactose at equilibrium in the reaction catalyzed by UDP-glucose 4-epimerase is 3.5 (5, 15). The preponderance of UDP-D-glucose in this mixture is probably due to the higher energy level of UDP-D-galactose associated with the axial hydroxyl at C-4. Since UDP-D-xylose and UDP-L-arabinose differ from their hexosyl analogues only in lacking a hydroxymethyl residue at C-5, one would expect the constant for the equilibrium between these compounds to be similar to that for the equilibrium between UDP-D-glucose and UDP-D-galactose. The unusually high proportion of UDP-L-arabinose present in the equilibrium mixture is at present unexplained and may be due to stabilization of the UDP-L-arabinose structure by association between the L-arabinosyl moiety and the uracil ring.

LITERATURE CITED