SC 11071

Thymidine diphosphate D-galactose pyrophosphorylase of *Phaseolus aureus*

Extracts of mung bean *(Phaseolus aureus)* seedlings have been shown to catalyze the formation of deTDP-D-galactose by the following reaction[1]:

deTTP + α-D-galactose 1-phosphate ⇌ deTDP-D-galactose + pyrophosphate

However, it was not determined whether this reaction was catalyzed by a distinct enzyme or by the highly active UDP-D-galactose pyrophosphorylase (UTP: α-D-galactose-1-phosphate uridylyltransferase, EC 2.7.7.10) present in the same preparations. Evidence presented in this communication shows that the pyrophosphorylase which forms deTDP-D-galactose (deTTP:α-D-galactose-1-phosphate thymidylyltransferase) is readily separable from that responsible for the synthesis of UDP-D-galactose. It is probable that deTDP-D-galactose pyrophosphorylase is also distinct from deTDP-D-glucose pyrophosphorylase (deTTP:α-D-glucose-1-phosphate thymidylyltransferase), although complete separation of the two has not been achieved. The presence of an enzyme specific for the synthesis (and breakdown) of deTDP-D-galactose suggests that this sugar nucleotide may play a role in the metabolism of the plant. It is possible that it may function as a specific galactosyl donor in the formation of some of the numerous oligo- and polysaccharides containing galactose.

Mung bean seedlings were germinated and ground as previously described[1]. The homogenate was centrifuged at 12 000 × *g* for 15 min, and the supernatant solution fractionated with solid ammonium sulfate between 0–30%, 30–60%, and

TABLE I

DISTRIBUTION OF PYROPHOSPHORYLASE ACTIVITY IN $(NH_4)_2SO_4$ FRACTIONS

The assay mixture consisted of 5 μmoles Tris buffer (pH 7.5), 1 μmole $MgCl_2$, 0.2 μmole UTP or deTTP, 0.2 μmole α-D-glucose 1-phosphate or α-D-galactose 1-phosphate and lyophilized mung bean enzyme. An excess of inorganic pyrophosphatase (pyrophosphate phosphohydrolase, EC 3.6.1.1) (2 μg of crystalline enzyme[2], obtained through the courtesy of Dr. K. K. REDDI) was included in the reaction mixture in order to hydrolyze the pyrophosphate formed. The mung bean enzyme was diluted sufficiently so that no more than 0.2 μmole P_i was released during incubation. The total volume of the reaction mixture was 0.13 ml. After 1 h at room temperature (25–26°), the reaction was stopped by heating for 1 min at 100°, and phosphate was measured by the method of LOWRY AND LOPEZ[3]. Where necessary, the protein precipitate was removed by centrifugation. The results were corrected for phosphate released from sugar phosphate (usually negligible) or nucleoside triphosphate (often considerable in crude extracts). Protein was measured by the method of LOWRY *et al.*[4].

Batch	*$(NH_4)_2SO_4$ fraction*	*Specific activity of pyrophosphorylase (μmoles P_i/h/mg protein)*		
		deTDP-D-galactose	*deTDP-D-glucose*	*UDP-D-galactose*
1	0–30	0.21		1.8
	30–60	0.95		1.7
	60–75	0.07		1.7
2	0–30	0.54	2.6	
	30–60	1.4	2.8	
	60–80	0.50	15.7	
3	30–60	5.3	1.8	
4	30–60	3.4	3.2	

60–80% satn. All operations were carried out at 0–4°. The precipitates were dissolved in a minimal volume of 0.01 M Tris buffer (pH 7.5) and dialyzed overnight against 5 l of 0.01 M mercaptoethanol. After dialysis, each fraction was treated with 0.1 vol. of 1 M $MnCl_2$. The precipitates were removed by centrifugation and the supernatant solutions dialyzed overnight against 5 l of 0.01 M mercaptoethanol, clarified by centrifugation, and lyophilized. The dry powders could be stored at −10° for several months without significant loss of activity. The distribution of pyrophosphorylase activity among the lyophilized fractions of some sample preparations is summarized in Table I. Since the 30–60% fraction is highest in deTDP-D-galactose pyrophosphorylase, it was used as starting material for further purification.

Attempts to separate UDP-D-galactose and deTDP-D-galactose pyrophosphorylases by differential adsorption to calcium phosphate or alumina C_γ gels were only partly successful. A clear-cut separation of the two enzymes, however, was obtained by the use of a DEAE-cellulose column (Fig. 1). All of the deTDP-D-galactose pyrophosphorylase was eluted below 0.1 M NaCl, while UDP-D-galactose pyrophosphorylase required higher concentrations of NaCl. Activity towards deUTP was observed in the fractions specific for the uridine but not the thymidine nucleotide.

The large variation *in ratio* of deTDP-D-galactose to deTDP-D-glucose pyrophosphorylase activity (Table I) suggests that these also are distinct enzymes. The 60–80% fraction, in particular, is much enriched in deTDP-D-glucose pyrophosphorylase. Furthermore, the relative activity in the 30–60% ammonium sulfate fractions varied considerably from batch to batch (from 0.5 to 3). However, several attempts to obtain deTDP-D-galactose pyrophosphorylase free of activity towards α-D-glucose 1-phosphate by the use of adsorbants resulted at best in a ratio of 2 to 1. This difficulty may in part be due to the instability of deTDP-D-galactose pyrophosphorylase in dilute solutions, half the activity being lost by overnight storage

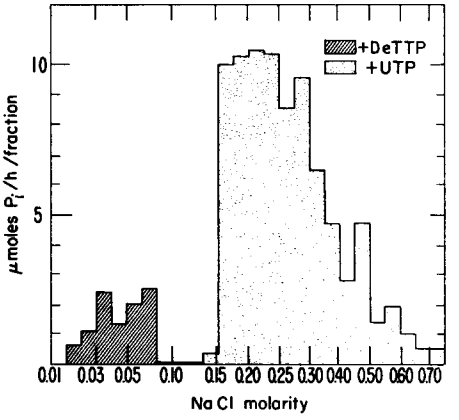

Fig. 1. Chromatography of deTDP-D-galactose and UDP-D-galactose pyrophosphorylases on DEAE-cellulose. The DEAE-cellulose (Eastman Chemicals) was treated sequentially with 1 N NaOH, water, 1 N HCl, and 0.01 M Tris buffer (pH 7.6). A solution of lyophilized 30–60% $(NH_4)_2SO_4$ fraction (22 mg protein in 1.1 ml of 0.01 M Tris, pH 7.6) was adsorbed on a column 13 × 0.8 cm. Elution was carried out with 0.01 M Tris buffer (pH 7.6) containing variable concentrations of NaCl as indicated. Fractions of 1–2.5 ml were collected and aliquots assayed as described in Table I. Since NaCl has no effect on the activity of the enzyme, the fractions were tested without preliminary dialysis.

at $-10°$. This is true of diluted solutions of the 30–60% ammonium sulfate fraction and of purified eluates from gels. The pyrophosphorylases for UDP-D-galactose and for deTDP-D-glucose are considerably more stable.

Optimal conditions for deTDP-D-galactose pyrophosphorylase activity were found to be 25–30°, pH 8.2 (in 0.04 M Tris buffer), and the presence of 3.3 mM $MgCl_2$ in the incubation mixture. Mn^{2+} and Co^{2+} were less effective than Mg^{2+}. *p*-Chloromercuribenzoate (10^{-4} M) inhibited the activity completely. Some nucleotides (UMP, deTMP, deUMP, and AMP) inhibited the reaction by 40–50% at a concentration of 1.5 mM; thymine or thymidine at the same concentration had no effect. K_m for α-D-galactose 1-phosphate was found to be 1.6 mM, and that for deTTP, 1 mM.

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[1] E. F. NEUFELD, *Biochem. Biophys. Res. Commun.*, 7 (1962) 461.
[2] M. KUNITZ, *J. Gen. Physiol.*, 35 (1952) 423.
[3] O. H. LOWRY AND J. A. LOPEZ, *J. Biol. Chem.*, 162 (1946) 421.
[4] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.

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Molecular weight of the phospho and dephospho forms of phosphoglucomutase

The molecular weight of phosphoglucomutase (D-glucose-1,6-diphosphate: D-glucose-1-phosphate phosphotransferase, EC 2.7.5.1) has a special importance because (a) it is a relatively high-molecular-weight protein which may have only one active site per molecule, (b) its molecular weight is of importance in determining the degree of phosphorylation, and (c) the phosphorylation and dephosphorylation of the enzyme might be accompanied by molecular-weight changes. KELLER, LOWRY AND TAYLOR[1] found a molecular weight of 74 000 using sedimentation and diffusion coefficient measurements on enzyme crystallized from ammonium sulfate[2]. The advent of more highly purified preparations of phosphoglucomutase[3,4] and the development by YPHANTIS[5] of a simple accurate equilibrium centrifugation method has led us to redetermine this value.

Crystalline phosphoglucomutase obtained by the method of NAJJAR[2] was chromatographed on carboxymethyl cellulose columns to obtain pure phospho and dephospho forms of the protein[3,4]. These forms were then tested in the Tiselius moving-boundary electrophoresis apparatus. Isoelectric points of both proteins were found to be near pH 7.0. There were, however, minor differences between the iso-