The Pyridine Nucleotide Requirement of Thymidine Diphosphate D-glucose and Cytidine Diphosphate D-glucose Oxidoreductases

Michio Matsuhashi,* Jeffrey M. Gilbert, Sachiko Matsuhashi,*
Joseph G. Brown and Jack L. Strominger*

Department of Pharmacology, Washington University
School of Medicine, St. Louis, Missouri

Received January 13, 1964

The formation of the 6-deoxy group of several hexoses occurs by an intramolecular oxidation-reduction accompanied by dehydration. A 4-keto-6-deoxyhexose is the product. These reactions occur while the sugars are linked to nucleotides. Thus, GDP-4-keto-6-deoxy-D-glucose is an intermediate in the formation of GDP-L-fucose from GDP-D-mannose (Ginsburg, 1961) and TDP-4-keto-6-deoxy-D-glucose is an intermediate in formation of TDP-L-rhamnose from TDP-D-glucose (Pazur and Shuey, 1961; Glaser and Kornfeld, 1961; Okazaki et al., 1962). In both cases formation of the nucleotide-linked 4-keto-6-deoxyhexose from the hexose had been reported to be stimulated 2-3 fold by DPN^+, suggesting that pyridine nucleotide might be an electron carrier in the intramolecular rearrangement. In the present paper purification and some properties of the enzyme which catalyzes the formation of TDP-4-keto-6-deoxy-D-glucose from TDP-D-glucose and of a newly discovered enzyme of this type which catalyzes formation of CDP-4-keto-6-deoxy-D-glucose from CDP-D-glucose will be reported.

*Present address: Department of Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin. Supported by research grants from the U. S. Public Health Service (A-1158) and National Science Foundation (G-18742).

TDP-D-glucose oxidoreductase has been purified 250-fold from a sonic extract of Escherichia coli strain B. TDP-4-keto-6-deoxy-D-glucose, the product of the reaction catalyzed by this enzyme, is a substrate for two pathways, formation of TDP-L-rhamnose and of TDP-4-acetamido-4,6-dideoxyhexose (Matsuhashi, 1963). The purified enzyme was specific for TDP-D-glucose as substrate and could not utilize TDP-D-galactose, TDP-D-mannose, CDP-D-glucose, dCDP-D-glucose, GDP-D-glucose, UDP-D-glucose or ADP-D-glucose. The reaction was irreversible. No requirement for or stimulation by pyridine nucleotide could be demonstrated. Moreover, the enzyme was obtained in an excellent overall yield with little loss of activity being encountered during purification. The purified enzyme was totally inhibited by p-chloromercuriphenylsulfonate (PCMP) and could not be reactivated by cysteine. Virtually full activity could be restored after inactivation by PCMP, however, by treatment with cysteine and DPN^+ (Table I). DPNH was also active, but the enzyme contained low

TABLE I

The effect of PCMP, cysteine and DPN^+ on activity of TDP-D-glucose oxidoreductase.

The reaction mixtures, containing 3.0 μg of purified enzyme (specific activity, 80 μmoles/mg/hr) and, if indicated, 8 mM PCMP in 40 μl of 0.1 M Tris HCl pH 7.6 were incubated for 8 min at 37°. Then when indicated, cysteine (final conc., 30 mM) and/or DPN^+ (final conc., 0.2 mM) were added. After incubation for 5 additional min 0.1 μmole of TDP-D-glucose was added and the reaction mixture was incubated for 20 min. The formation of TDP-4-keto-6-deoxy-D-glucose was measured by formation of absorption at 320 mμ in 0.1 N NaOH (final vol., 640 μl).

Conditions	Δ $A_{320\ m\mu}$
Untreated Enzyme	0.560 (0.08 μmole)
+ PCMP	0.002
+ PCMP + Cysteine	0.012
+ PCMP + DPN^+	0.003
+ PCMP + Cysteine + DPN^+	0.490
+ PCMP + Cysteine + DPN^+*	0.437

*In this case TDP-D-glucose was added before the second incubation and the reaction was started by adding DPN^+.

DPNH oxidase activity. TPN^+ and TPNH were totally inactive (Table II). Thus, it seems likely that, as in the case of glyceraldehyde phosphate dehydrogenase (Velick, 1958; Racker and Krimsky, 1958) and yeast UDP-D-glucose epimerase (Maxwell and de Robichon-Szulmajster, 1960), the purified enzyme contains bound DPN^+ which is released by PCMP and is essential for enzyme activity.[1]

CDP-D-glucose oxidoreductase was purified 200-fold from a sonic extract of Pasteurella pseudotuberculosis, Type V. The product of the reaction catalyzed by this enzyme, CDP-4-keto-6-deoxy-D-glucose, has been isolated and characterized, by methods previously used for characterization of TDP-4-keto-6-deoxy-D-glucose (Okazaki et al., 1962).[2] Like TDP-4-keto-6-deoxy-D-glucose it has an absorption maximum in alkali at 318 mμ with a molar extinction coefficient of about 5,000. It is an intermediate in the biosynthesis of CDP-3,6-dideoxy-L-mannose (CDP-ascarylose) (see accompanying communication). In contrast to TDP-D-glucose oxidoreductase, CDP-D-glucose oxidoreductase had an absolute requirement for DPN^+ from the first fractionation step (Table II). DPNH was again active, but TPN^+ and TPNH were inactiv This enzyme could also be inactivated by PCMP, but could not be reactivated by cysteine and DPN^+. The purified CDP-D-glucose oxidoreductase was specific for CDP-D-glucose or dCDP-D-glucose as substrat and could not utilize TDP-D-glucose, TDP-D-galactose, TDP-D-mannose, CDP-D-galactose, CDP-D-mannose, GDP-D-glucose, GDP-D-mannose, UDP-D-glucose or ADP-D-glucose. No reversibility could be demonstrated,

---

[1] Dr. Sidney Velick suggested this maneuver to remove bound DPN^+ to us.

[2] The isolated compound contained 2.06 moles of organic phosphate and 0.95 mole of reducing group (based on fericyanide reduction) per mole of cytidine (λ max = 280 mμ in acid, $\frac{A280}{A260}$ = 2.15). The sugar had the same paper chromatographic mobilities as the sugar from TDP-4-keto-6-deoxy-D-glucose. It gave a positive O-phenylenediamine reaction. After hydrogenation with rhodium catalyst, it yielded fucose and 6-deoxyglucose. The fucose did not react with L-fucose isomerase and hence was presumably D-fucose. The 6-deoxyglucose was oxidized by D-glucose oxidase and hence was 6-deoxy-D-glucose.

TABLE II

Requirement of purified TDP-D-glucose and CDP-D-glucose oxidoreductases for pyridine nucleotides

Nucleotides	TDP-glucose oxidoreductase*		CDP-glucose oxidoreductase**	
	Conc. of cofactor, M	% Activity of PCMP-cysteine treated enzyme	Conc. of cofactor, M	% Activity
DPN^+	1.10^{-3}	44		
	2.10^{-4}	33	1.10^{-4}	(100)
	2.10^{-5}	10	1.10^{-5}	100
	2.10^{-6}	5	1.10^{-6}	86
			1.10^{-7}	21
DPNH	1.10^{-3}	38		
	2.10^{-4}	33	1.10^{-4}	69
	2.10^{-5}	12	1.10^{-5}	72
	2.10^{-6}	3	1.10^{-6}	68
			1.10^{-7}	22
TPN^+	2.10^{-4}	3	1.10^{-4}	21
TPNH	2.10^{-4}	1	1.10^{-4}	12
None	--	3	None	4

*The conditions are same as in Table I except the first incubation was carried out with 5 mM PCMP for 15 min at 37° followed by a 5 min incubation with cysteine and cofactor and a 60 min incubation with TDP-D-glucose. One µg of enzyme protein was used with a specific activity of 62 µmoles/mg/hr. Data are expressed as % of activity of untreated enzyme. In this experiment only 44 % reactivation was obtained as a maximum, in contrast to the experiment of Table I, because of the longer time of exposure to PCMP.

**The reaction mixtures, containing 3.1 µg of purified CDP-D-glucose-oxidoreductase (specific activity, 14 µmoles/mg/hr), 0.05 M of Tris HCl pH 7.5, 32 mµmoles of CDP-D-glucose and cofactor as indicated (final vol., 0.1 ml), were incubated for 20 min at 37°. The formation of CDP-4-keto-6-deoxy-D-glucose was measured by formation of absorptic at 320 mµ in alkali.

even with CDP-4-keto-6-deoxy-D-glucose of high specific radioactivity. The crude extract also contained TDP-D-glucose oxidoreductase but this enzyme was not detectable after purification.

These experiments demonstrate that TDP-D-glucose and CDP-D-glucose oxidoreductases are separate enzymes and that both of them have absolute requirements for DPN^+. In one case, however, the DPN^+ remained firmly bound to the enzyme, while in the other case

it was readily removed during purification. Further experiments will be needed to establish the precise role of DPN^+, but the nature of the reactions catalyzed by these enzymes certainly suggests a role of the coenzyme in an intramolecular transfer of protons and electrons from C-4 to C-6.

REFERENCES

Ginsburg, V., J. Biol. Chem., 236, 2389 (1961).
Glaser, L., and Kornfeld, S., J. Biol. Chem., 236, 1795 (1961).
Matsuhashi, M., Federation Proc., 22, 465 (1963).
Maxwell, E. S., and deRobichon-Szulmajster, H., J. Biol. Chem., 235, 308 (1960).
Okazaki, R., Okazaki, T., Strominger, J. L., and Michelson, A. M., J. Biol. Chem., 237, 3014 (1962).
Pazur, J. H., and Shuey, E. W., J. Biol. Chem., 236, 1780 (1961).
Racker, E., and Krimsky, I., Federation Proc., 17, 1135 (1958).
Velick, S. F., J. Biol. Chem., 233, 1455 (1958).