THE MECHANISM OF 6-DEOXYHEXOSE SYNTHESIS IV.

THE ROLE OF PYRIDINE NUCLEOTIDE IN SUBSTRATE RELEASE[1]

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Summary: Evidence is presented to show that in the dTDP-D-glucose oxidoreductase reaction, product is only released from enzyme containing DPN, and product release from enzyme containing DPNH does not occur at a significant rate. This result is interpreted to indicate that the conformation of the protein depends on the oxidation state of the bound pyridine nucleotide.

One of the puzzling features of reactions like the dTDP-D-glucose oxidoreductase (Melo et al., 1968; and Gabriel and Linquist, 1968) or the UDP-D-glucose-4'-epimerase, is the fact that intermediates which are structurally very similar to both the substrate and the product of the reaction remain tightly bound to the enzyme. This could result from the covalent binding of these intermediates to the enzyme or conformational changes in the enzyme during catalysis, such that substrate release is not possible.

The dTDP-D-glucose oxidoreductase catalyzes the following reaction [where E = enzyme].

$$\begin{matrix} E \\ | \\ DPN \end{matrix} + \text{dTDP-Glu} \rightleftharpoons \begin{matrix} E\text{-dTDP-Glu} \\ | \\ DPN \end{matrix} \rightleftharpoons \left[\begin{matrix} E\text{-dTDP-4-keto-glucose} \\ | \\ DPNH \end{matrix} \right] \xrightarrow[H_2O]{} \qquad \text{Eq.1}$$

$$\left[\begin{matrix} E\text{-dTDP-xylo-4-hexulose,5,6 een} \\ | \\ DPNH \end{matrix} \right] \rightarrow \begin{matrix} E\text{-dTDP-4-keto-6-deoxyglucose} \\ | \\ DPN \end{matrix} \quad \uparrow\downarrow \quad \begin{matrix} E + \text{dTDP-4-keto-6-deoxyglucose} \\ | \\ DPN \end{matrix}$$

where intermediates in brackets exist only as enzyme bound intermediates. In this communication we present evidence that when dTDP-4-keto-6-deoxyglucose, the normal product of the reaction, is formed

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on the enzyme by oxidation of dTDP-6-deoxyglucose, to yield E-DPNH-dTDP-4-keto-6-deoxyglucose it cannot dissociate from the enzyme, thus providing evidence that E-DPNH has a different conformation from E-DPN such that substrate cannot readily dissociate from it. Evidence of a very different kind has been provided by Bertland and Kalckar (1968) to show that with yeast UDP-glucose-4'-epimerase, the conformation of the protein is changed by reduction of enzyme bound DPN, although the effect of this change on substrate release is not known.

MATERIALS AND METHODS: dTDP-D-glucose oxidoreductase was prepared as described previously (Zarkowsky and Glaser, 1969). Enzyme containing bound DPN ^{3}H was prepared as described in the legend of Fig. 1.

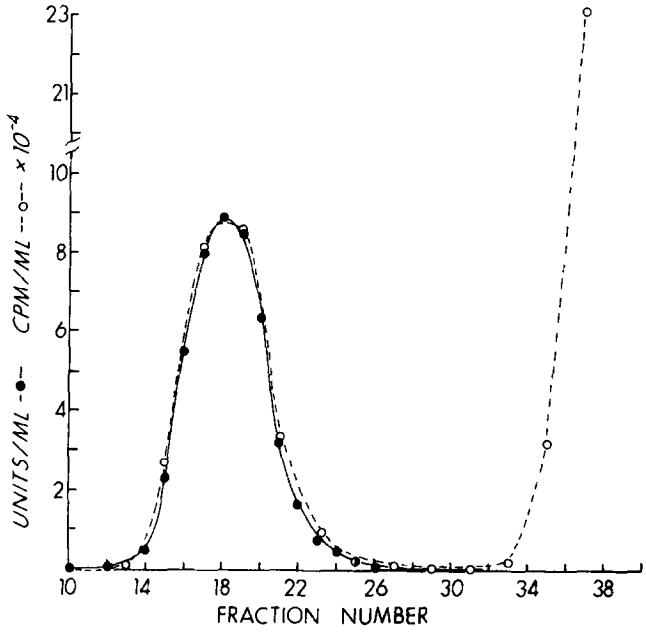

Fig. 1: Separation of enzyme-DPN ^{3}H from DPN ^{3}H on Sephadex G-100. To 0.4 ml of a solution containing 42 units enzyme, 15 μmoles of Tris-Cl, pH 8.0, 30 μmoles of potassium phosphate, pH 8.0 were added 0.15 ml of glycerol and 0.06 ml of 5 x 10^{-2}M p-hydroxymercuribenzoate; after incubation at 30° for 20 min., 0.02 ml of 0.75 M 2-mercaptoethanol were added; after 20 min. at 25° 0.1 ml of DPN ^{3}H (New England Nuclear) (5μC) were added and incubation at 25° was continued for 15 min. The enzyme was separated from free DPN by chromatography on a 1 x 50 cm column of Sephadex G-100 equilibrated with 0.025 M 2-mercaptoethanol in 0.025 M Tris-Cl, pH 8.0 and eluted with the same buffer. 1 ml fractions were collected. The recovery of enzyme in different experiments varied between 70 and 90%.

In order to determine enzyme bound DPNH, enzyme containing DPN ^{3}H was incubated with the additions indicated in individual experiments at 16° in a total volume of 0.7 ml. The reaction was terminated by the addition of

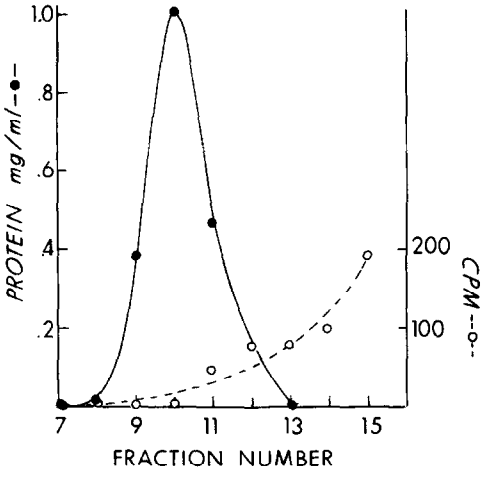

Fig. 2: Separation of enzyme and DPN ^{3}H after alkaline denaturation. To labelled enzyme 7210 CPM in 0.7 ml of 0.05 M Tris-Cl, pH 8.0 containing 2 mg of serum albumin were added 0.3 ml of the quench solution and chromatographed on a 1 x 30 cm Sephadex G-25 column in 0.1% Tris base, 0.8 ml fractions were collected.

0.3 ml of freshly prepared 0.14 M NaOH containing 1 μmole of DPNH, 1 μmole of DPN, 0.6 mg of sodium dodecyl sulfate (quench solution). This addition released all the bound pyridine nucleotide from the enzyme (Fig. 2). In order to separate the DPN from DPNH, the reaction mixtures were chromatographed as described in legend to Fig. 3.

A mixture of dTDP-6-deoxy-D-glucose and dTDP-6-deoxy-D-galactose (dTDP-6dGlu/6dGal) was prepared by $NaBH_4$ reduction of dTDP-4-keto-6-deoxy-D-glucose and purified by chromatography on DEAE Sephadex A-25 eluted with a gradient of triethylammonium acetate. dTDP-6-deoxyglucose (dTDP-6dGlu) was separated from dTDP-6dGal by paper chromatography (Carminatti and Passeron, 1966). Nucleotides eluted from the paper were further purified by adsorbtion and elution from Darco G-60.

RESULTS AND DISCUSSION: The data in Fig. 3 and Table 1 demonstrates that on addition of dTDP-Glu, dTDP-6dGlu/6dGal or dTDP-6dGlu, the formation of enzyme bound DPNH can be demonstrated. The percentage of enzyme bound DPN reduced by each of these substrates or substrate analogues has varied with different enzyme preparations, suggesting that some of the protein bound DPN ^{3}H may be on inactive enzyme molecules. However, as illustrated in Table 1, if the data are normalized to the amount of DPNH formed on the addition of dTDP-D-glucose, the results

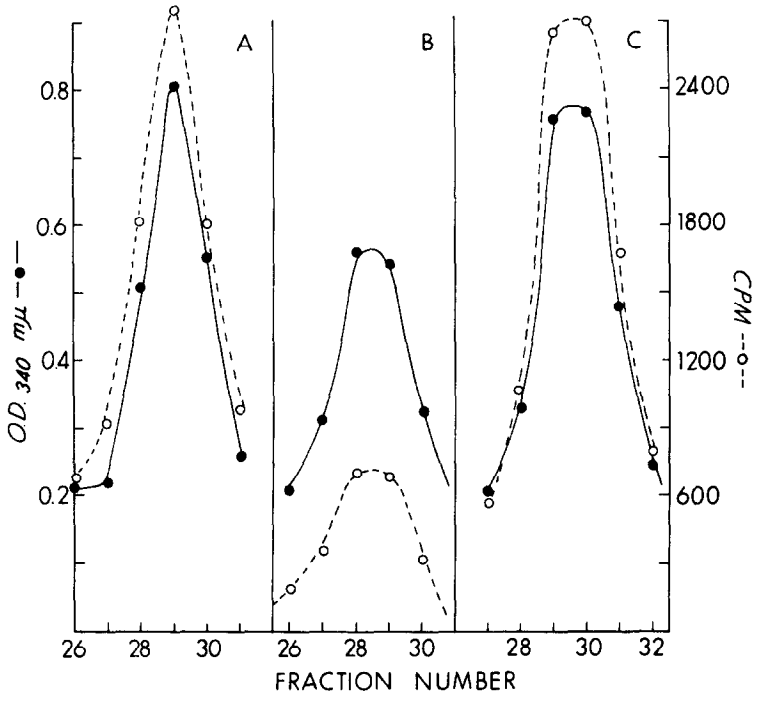

Fig. 3: Formation of radioactive DPNH on addition of substrate analogues. The reaction mixture in A contained 50 μmoles of Tris-Cl, pH 8.0, 1.1 units of enzyme (26,600 CPM) and 1 μmole of dTDP-6dGlu/6dGal. After incubation at 16° for 1 min., the reaction was stopped with 0.3 ml of quench solution and chromatographed on a 1 x 24 cm column of DEAE Sephadex A-25 equilibrated with 0.1% Tris base. The column was washed with 0.1 M NaCl in 0.1% Tris base until all the DPN had been eluted. DPNH was then eluted with 0.25 M NaCl in 0.1% Tris base. 1.5 ml fractions were collected. Shown in the drawing is the elution of DPNH with 0.25 M NaCl in 0.1% Tris base. The absorbancy at 340 mμ is from carrier DPNH, the radioactivity is from the enzyme. In B, an identical incubation was set up, but after 1 min., 1 μmole of dTDP-Glu was added and incubation continued for 30 sec. In C, the same incubation as in A was set up. After 1 min., 0.01 μmole of dTDP-4-keto-6-deoxyglucose was added and after 30 sec. the reaction stopped with 0.3 ml of quench solution.

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are essentially the same with all enzyme preparations. It should be mentioned that Gabriel and Wang (1969) isolated tritium labelled DPNH by reduction of enzyme bound DPN by dTDP-6dGlu ^{4}H Reduction by dTDP-6dGal does not occur. The data in Fig. 4 shows that dTDP-6dGlu/6dGal is a competitive inhibitor of the dTDP-D-glucose oxidoreductase.

The fact that enzyme bound DPNH is produced upon the addition of dTDP-6dGlu shows that the enzyme can catalyze the reaction.

$$\text{dTDP-6dGlu} + \underset{\text{DPN}}{\text{E}} \rightleftharpoons \underset{\text{DPN}}{\text{E-dTDP-6dGlu}} \rightleftharpoons \underset{\text{DPNH}}{\text{E-dTDP-4-keto-6dGlu}} \qquad \text{Eq. 2}$$

TABLE 1

REDUCTION OF ENZYME BOUND DPN

The experiments were carried out as described in Materials and Methods and Fig. 3. In experiment I, 1.13 units of enzyme containing 26,670 CPM of DPN ^{3}H were used per reaction mixture. In experiment II a different enzyme preparation was used with 0.75 units of enzyme containing 26, 170 CPM of DPN ^{3}H per reaction mixture. All experiments were carried out at 16°. 1 unit of enzyme (Zarkowsky and Glaser, 1969) will catalyze the formation of 4.1 mμmoles of product in the standard assay per minute at 16°. First incubations were for 1 min. Second incubations for 30 sec. Numbers in parentheses are micromoles of substrates added.

Expt.		First Incubation	Second Incubation	DPNH CPM	DPNH Relative to dTDP-Glu
	A	None	None	0	0
	B	dTDP-Glu (0.1)	-	1,580	1
	C	dTDP-6dGlu/6dGal* (1.0)	-	15,400	9.7
	D	dTDP-6dGlu/6dGal* (1.0)	dTDP-Glu (1.0)	5,100	3.2
I	E	dTDP-6dGlu/6dGal* (1.0)	dTDP-4-keto-6dGlu (.01)	16,100	10.2
	F	dTDP-6dGlu/6dGal (1.0)	-	15,800	10.0
	G	dTDP-6dGlu/6dGal (1.0)	dTDP-Glu (1.0)	7,100	4.5
	H	dTDP-6dGlu/6dGal (1.0)	dTDP-4-keto-6dGlu (.01)	15,200	9.6
	I	dTDP-6dGlu/6dGal (0.1)	-	5,920	3.7
	J	dTDP-6dGlu/6dGal (0.1)	dTDP (1)	0	0
	A	dTDP-Glu (0.1)	-	730	1
	B	dTDP-6dGlu (0.7)*	-	6,800	9.3
	C	dTDP-6dGlu (0.7)*	dTDP-Glu (1.0)	2,080	2.97
II	D	dTDP-6dGlu (0.7)		7,580	10.4
	E	dTDP-6dGlu (0.7)	dTDP-Glu (1.0)	1,800	2.5
	F	dTDP-6dGlu (0.07)		4,560	6.3
	G	dTDP-6dGlu (0.07)	dTDP (1.0)	0	0

* 5 minute incubation

Clearly the dehydration step in the normal reaction sequence cannot occur with this substrate analogue, nor have we been able to demonstrate an exchange of the hydrogen at C-5 of the dTDP-6dGlu with the solvent. The question of interest is whether dTDP-4-keto-6dGlu can dissociate from E-DPNH to yield an inactive enzyme.

The data in Table 1 shows that the reduction of DPNH by

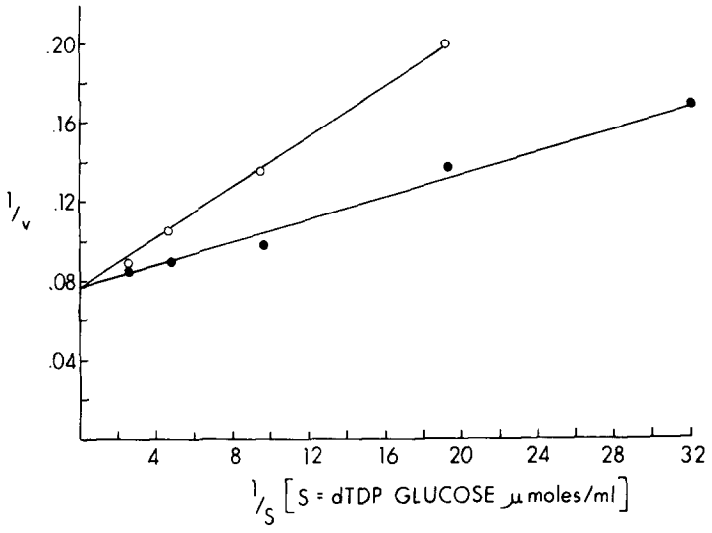

Fig. 4: Competitive inhibition of dTDP-glucose oxidoreductase by dTDP-6dGlu/6dGal. Standard assay conditions were used at 37° as described previously (Zarkowsky and Glaser, 1969). -●- no additions. -○- with 0.20 μmoles/ml of dTDP-6dGlu/6dGal.

dTDP-6dGlu can be reversed by dTDP and by dTDP-Glu, and furthermore that the effect of dTDP-Glu is not due to small quantities of dTDP-4-keto-6dGlu formed during the incubation, since addition of the maximum quantity of dTDP-4-keto-6dGlu that could be formed under these conditions has no effect (Expt. IE and IH).

Clearly this observation cannot be explained if dTDP-4-keto-6dGlu dissociates from E-DPNH, because then reoxidation of E-DPNH could only be observed if large quantities of dTDP-4-keto-6dGlu were added to the enzyme. The observation can be explained by the fact that steps 1 and 2 - Eq. 2 are reversible and dTDP-Glu and dTDP can compete with dTDP-6dGlu for binding to the enzyme and that substrate cannot dissociate from E-DPNH. The fact that dTDP-Glu only leads to a partial reoxidation of E-DPNH under conditions of Table 1, is expected from the fact that under the conditions used there is present an equimolar quantity of dTDP-Glu and dTDP-6dGlu/6dGal and that the K_i for dTDP-6dGlu/6dGal (3×10^{-4} M) is only six fold larger than the K_m for dTDP-Glu (5×10^{-5} M) (Fig. 4).

It should be noted that under the conditions used, even a slow dissociation of substrate from E-DPNH seems not to take place, since essentially the same results were obtained (Table 1) with 1 and 5 min. incubation

periods with the substrate analogue. Similarly we have incubated enzyme with 1 mM dTDP-6dGlu at 16° for 20 min., and noted no loss of enzyme activity when assayed with excess dTDP-Glu.

Experiments to try to directly demonstrate a change in protein conformation on reduction of pyridine nucleotide are in progress.

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