The Nucleotide Specificity and Feedback Control of Thymidine Diphosphate d-Glucose Pyrophosphorylase*

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A large number of nucleotide diphosphate sugar pyrophosphorylases have been described (for review see References 1 and 2), but only a few of these have been purified to any significant extent. In this paper we report the further purification of deoxythymidine diphosphate n-glucose pyrophosphorylase from Pseudomonas aeruginosa (3). With this partially purified enzyme, it is possible to show that this enzyme will utilize not only thymidine nucleotides as substrates but also uridine nucleotides and deoxyuridine nucleotides.

The control of the enzymatic reactions involved in the biosynthesis of structural polysaccharides has only recently become a subject of investigation. In particular, Kornfeld et al. (4) have shown that UDP-GlcNAc is a feedback inhibitor of the n-fructose 6-phosphate l-glutamine amidotransferase, and also that CMP-N-acetyleneuraminic acid is an inhibitor of the enzyme that cleaves UDP-GlcNAc to N-acetyl-n-mannosamine. In each case the end product inhibits the first enzyme that is unique to its biosynthetic pathway.

It is shown in this paper that the dTDP-n-glucose pyrophosphorylase is subject to feedback inhibition by dTDP-L-rhamnose. In an accompanying paper, Bernstein and Robbins (5) describe related observations obtained independently on the dTDP-n-glucose pyrophosphorylase and the UDP-n-glucose pyrophosphorylase from Salmonella.

EXPERIMENTAL PROCEDURE

Materials and Methods

Alumina gel Cy was prepared by the method of Willstätter, Kraut, and Erbacher (6). It contained 15 mg per ml (dry weight) and had been aged at 3° before use. Nucleotide triphosphates, UDP-n-glucose, and crystalline phosphoglucomutase were products of the Sigma Chemical Company. Glucose 6-phosphate dehydrogenase was obtained from Boehringer and Soehne. Inorganic pyrophosphatase was obtained from the Worthington Biochemical Corporation. One unit of each of these auxiliary enzymes is defined as 1 μmole of product formed per minute. Sephadex G-200 was obtained from Pharmacia Fine Chemicals, Inc.

Protein was determined by the method of Warburg and Christian (7) or by the method of Lowry et al. (8). All radioactivity measurements were carried out in a Nuclear-Chicago Corporation thin window counter with a background of 1.5 to 2.0 c.p.m.

dTDP-d-glucose was prepared enzymatically as described previously (3) with the heat-treated enzyme described below. dUDP-n-glucose was prepared by the same method with dUTP as a substrate. Commercial dTDP-n-glucose (Calbiochem) was used in some experiments. dTDP-L-rhamnose was prepared by the method of Glaser and Kornfeld (9). When extracts of P. aeruginosa ATCC 1419 were used, it was found necessary to omit MgCl₂ from the incubation mixture to prevent enzymic hydrolysis of dTDP-L-rhamnose. The dTDP-L-rhamnose was isolated by chromatography on Dowex 1-X8-formate (10), α-D-Glucose-14C-1-P was prepared from starch and crystallized as the K⁺ salt. It had a specific activity of 52,000 c.p.m per μmole.

dTTP labeled with 32P in the two terminal phosphate groups was prepared from dTDP-d-glucose and P32Pi in the presence of purified dTDP-d-glucose phosphophosphorylase, phosphoglucomutase, and α-glucose 1,6-diphosphate, isolated by chromatography in ethanol-1 M ammonium acetate, pH 7.8 (7.5:1) (11), and further purified by adsorption and elution from charcoal. α-D-Glucose 1,6-diphosphate was a kind gift of Dr. H. Narahara.

Assay of dTDP-n-glucose Pyrophosphorylase

Three assay methods were used.

Assay 1: Pyrophosphorylases of dTDP-d-glucose—This assay has been used previously (3). The reaction mixtures contained 100 μmoles of Tris-Cl, pH 8.0, 10 μmoles of MgCl₂, 0.5 μmole of TPN, 0.002 μmole of α-glucose 1,6-diphosphate, 0.2 unit of phosphoglucomutase, and 0.2 unit of glucose-6-P dehydrogenase in volume of 1 ml. Reactions were carried out in a Beckmann DU spectrophotometer, thermostatically controlled at 25°, in cells of 1-cm light path. One unit of enzyme is defined as the quantity of enzyme catalyzing an optical density change of 1.0 per minute in this assay when carried out with 1 × 10⁻⁴ M dTDP-d-glucose and 5 × 10⁻⁴ M PP₁ as substrates.

Assay 2: Synthesis of dTDP-d-glucose from α-D-glucose-14C-1-P and Nucleotide Triphosphate—The reaction mixtures contained 100 μmoles of Tris-Cl, pH 8.0, 10 μmoles of MgCl₂, 0.12 unit of inorganic pyrophosphatase, α-D-glucose-14C-1-P, and nucleotide triphosphate as indicated in the individual experiments in a...
The purifications of dTDP-glucose pyrophosphorylase

**Table I**

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity</th>
<th>Total units</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 105,000 X g</td>
<td>310</td>
<td>18.0</td>
<td>0.40</td>
<td>2500</td>
</tr>
<tr>
<td>2. Protamine sulfate + 40-60% (NH₄)₂SO₄</td>
<td>25.3</td>
<td>35.8</td>
<td>1.95</td>
<td>1770</td>
</tr>
<tr>
<td>3. Heat treatment</td>
<td>24</td>
<td>13.4</td>
<td>5.6</td>
<td>1800</td>
</tr>
<tr>
<td>4. Alumina gel Cy³</td>
<td>11 (10)</td>
<td>1.7 (1.8)</td>
<td>16.0 (14.4)</td>
<td>297 (200)</td>
</tr>
<tr>
<td>5. Ammonium sulfate + Sephadex S-200 chromatography</td>
<td>4 (3.8)</td>
<td>0.45 (0.54)</td>
<td>90 (78)</td>
<td>162 (160)</td>
</tr>
</tbody>
</table>

*Steps 4 and 5 were carried out on 5-ml aliquots of the enzyme from Step 3. The values in parentheses indicate a second run with the same enzyme.*

The remaining enzyme was recovered in fractions of lower specific activity.

The purifications of dTDP-glucose pyrophosphorylase

**P. aeruginosa** (ATCC 14149) was grown as described by Hauser and Kasowsky (13). Cell-free extracts were prepared as described previously (3), except that the time for cell rupture in the sonic oscillator was increased to 30 minutes, and the small particles were removed by centrifugation at 105,000 X g for 4 hours. The supernatant fluid obtained after the removal of the small particles was used as a starting material for the enzyme purification.

The supernatant fluid, from the high speed centrifugation, was added enough freshly prepared 2% protamine sulfate to bring the ratio of A₂₆₀ : A₂₈₀ to 0.8. After 20 minutes, the precipitate was removed by centrifugation, and the supernatant fluid was fractionated with a neutral ammonium sulfate.

The fraction precipitating between 40 to 60% saturation was collected by centrifugation at 12,000 X g for 15 minutes and dissolved in a minimal volume of 0.05 M Tris-Cl 0.01 M MgCl₂, 0.001 M EDTA, pH 8.0, and then dialyzed for 6 hours against six successive 1-liter portions of the same buffer.

To the dialyzed solution was added enough dTTP to bring the final concentration to 1 X 10⁻³ M, and the solution was kept in a 60° water bath for 20 minutes. After cooling to 0°, denatured protein was removed by centrifugation at 12,000 X g for 10 minutes.

The supernatant fluid from the heat step was diluted with an equal volume of 0.05 M Tris-Cl 0.01 M MgCl₂, 0.001 M EDTA, pH 8.0, and 0.14 ml of alumina gel Cy added per mg of protein. After 30 minutes, the gel was removed by centrifugation at 2,000 X g for 10 minutes, washed by centrifugation with 0.05 M Tris-Cl 0.001 M MgCl₂, 0.001 M EDTA, pH 8.0, and eluted with 0.02 M potassium phosphate-0.001 M EDTA, pH 7.5 (a volume equal to the original volume of enzyme was used). In some cases the recovery of enzyme was low, and the remaining enzyme could be recovered with a second elution of the gel.

The gel eluate was brought to 60% saturation with ammonium sulfate, and after 1 hour, the precipitate was collected by centrifugation at 12,000 X g for 20 minutes and suspended in a minimal volume of 0.05 M Tris-Cl 0.01 M MgCl₂, 0.001 M EDTA, pH 8.0. The enzyme was then chromatographed on a column of Sephadex G-200 (1 X 100 cm) equilibrated with the same buffer. Fractions (9 ml) were collected at a rate of one fraction every 2 hours. Enzyme was eluted in Fractions 17 to 25.

A summary of the purification procedure is shown in Table I. This purification has been reproducible for six separate complete preparations. The enzyme obtained by this procedure is about 20 times more active than preparations obtained previously. The calcium phosphate gel step in the previously published procedure has not been reproducible with different gel preparations. The enzyme is free of inorganic pyrophosphatase, phosphoglucomutase, and enzymes catalyzing the breakdown of glucose-1-phosphate.

All steps in the enzyme purification, except where specifically indicated, were carried out at 0°.

Ammonium sulfate was saturated at 0° and neutralized with NH₄OH so that a 0.20 dilution gave a pH of 7.5 when read at room temperature in a Beckmann pH meter.

*In previous work with this enzyme (3) a different strain of Pseudomonas aeruginosa ATCC 7700 was used. No significant differences between the enzymes of these two strains have been detected.*
The enzyme is stable when stored in a frozen state after the heat treatment. The enzyme obtained from the Sephadex G-200 column, when kept frozen, lost about 30% of the activity in 1 month.

Properties of dTDP-D-glucose Pyrophosphorylase

Nucleotide Specificity—The purified enzyme will utilize uridine and deoxyuridine nucleotides in place of thymidine derivatives. Adenosine, guanosine, and cytosine triphosphates, as well as ADP-D-glucose, CDP-D-glucose, and GDP-D-glucose, are not substrates for the enzyme. That these activities are all catalyzed by the same enzyme is supported by the following observations.

The enzyme is specifically protected against thermal inactivation by the addition of dTTP or dTDP-D-glucose. In the 105,000 × g supernatant fluid only 50% of the total UDP-D-glucose pyrophosphorylase activity is protected from inactivation by the presence of dTTP (Fig. 1A). In the fraction precipitated between 40 to 60% saturation with ammonium sulfate and all more purified fractions, all of the apparent UDP-D-glucose pyrophosphorylase activity is protected against thermal denaturation by thymidine nucleotides. We interpret these observations to indicate that in the crude extract about 50% of the apparent UDP-glucose pyrophosphorylase activity is catalyzed by the dTDP-D-glucose pyrophosphorylase. This also agrees with the observation (see below) that in the supernatant fluid (105,000 × g), TDP-D-rhamnose will only block 50% of the apparent UDP-D-glucose pyrophosphorylase activity, but will block it completely in the ammonium sulfate fraction and all more purified fractions. The ratio of dTDP-D-glucose pyrophosphorylase to UDP-D-glucose pyrophosphorylase from the ammonium sulfate fraction to the Sephadex G-200 eluate remains constant. The effects of various substrates on the stability of the enzyme at 60° are listed in Table II. Uridine or deoxyuridine nucleotides at

Table II

<table>
<thead>
<tr>
<th>Additions</th>
<th>Half-life (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>6.0</td>
</tr>
<tr>
<td>3 × 10⁻⁴ M glucose-1-P</td>
<td>6.5</td>
</tr>
<tr>
<td>5 × 10⁻⁴ M PP₃</td>
<td>0.5</td>
</tr>
<tr>
<td>8 × 10⁻⁵ M dTDP-glucose</td>
<td>8</td>
</tr>
<tr>
<td>2.5 × 10⁻⁵ M dTDP-glucose</td>
<td>22</td>
</tr>
<tr>
<td>5 × 10⁻⁴ M dTDP-glucose</td>
<td>31</td>
</tr>
<tr>
<td>3 × 10⁻⁴ M dTDP</td>
<td>15</td>
</tr>
<tr>
<td>Experiment B</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>8.0</td>
</tr>
<tr>
<td>1 × 10⁻³ M UTP</td>
<td>9.0</td>
</tr>
<tr>
<td>1 × 10⁻³ M UDP-D-glucose</td>
<td>8.0</td>
</tr>
<tr>
<td>1 × 10⁻³ M DTTP</td>
<td>30</td>
</tr>
<tr>
<td>Experiment C</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>4.5</td>
</tr>
<tr>
<td>1 × 10⁻⁴ M DTTP</td>
<td>6.0</td>
</tr>
<tr>
<td>3 × 10⁻⁴ M DTTP</td>
<td>11.0</td>
</tr>
<tr>
<td>6 × 10⁻⁴ M DTTP</td>
<td>16.0</td>
</tr>
<tr>
<td>4 × 10⁻⁴ M dUTP</td>
<td>4.5</td>
</tr>
<tr>
<td>Experiment D</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>5.0</td>
</tr>
<tr>
<td>5 × 10⁻⁵ M dTDP-D-rhamnose</td>
<td>5.0</td>
</tr>
<tr>
<td>1 × 10⁻⁴ M dTDP-D-rhamnose</td>
<td>6.0</td>
</tr>
</tbody>
</table>

![Fig. 1](image-url)  
**Fig. 1.** The stability of dTDP-D-glucose pyrophosphorylase. In Fig. 1A the 105,000 × g supernatant fluid was heated at 60° for varying periods of time, and after removal of denatured protein by centrifugation, the residual activity was determined with Assay 1, with either UDP-D-glucose or dTDP-D-glucose as a substrate. In Fig. 1B the fraction precipitated between 40 and 60% saturation with ammonium sulfate. ●, dTDP-D-glucose pyrophosphorylase activity when enzyme is heated in the presence of 1 × 10⁻⁴ M dTTP; ○, UDP-D-glucose pyrophosphorylase activity in the presence of 1 × 10⁻⁴ M dTTP; ▲, dTDP-D-glucose pyrophosphorylase activity with no additions; Δ, UDP-D-glucose pyrophosphorylase activity with no additions.

![Fig. 2](image-url)  
**Fig. 2.** Effect of nucleotide triphosphates on the pyrophosphorylysis of dTDP-D-glucose. Assay 1 was used with concentrations of dTDP-D-glucose as indicated and enzyme (specific activity, 40 units per mg of protein). In A, ● represents no addition; ◊, 4 × 10⁻⁴ M dTTP; ○, 6 × 10⁻⁴ M dTTP; ▲, 1 × 10⁻³ M dTTP. In B, ● represents no inhibitor; ◊, 2 × 10⁻⁴ M dUTP; ○, 4 × 10⁻⁴ M dUTP; r represents micromoles of product formed per minute.
the concentration tested in Table II have no effect on the stability of the enzyme.

Both the dTDP-β-glucose pyrophosphorylase activity as well as the UDP-β-glucose pyrophosphorylase activity are inhibited by dTTP and dUTP (Figs. 2 to 4). UTP shows a slight inhibitory effect with both substrates at concentrations greater than 10^{-3} \text{M} and has not been investigated further. The \( K_i \) for dTTP in the dTDP-β-glucose pyrophosphorylase reaction (from Fig. 2) is 2.6 \times 10^{-3} \text{M} and that for dUTP is 2.6 \times 10^{-4} \text{M}. The corresponding values for the UDP-β-glucose pyrophosphorylase reaction are 1 \times 10^{-4} \text{M} for dTTP and 1 \times 10^{-3} \text{M} for dUTP (Fig. 3).

The inhibition by the deoxynucleotide triphosphates is competitive with respect to the sugar nucleotide and uncompetitive with PP_i (Fig. 4).

Fig. 5A illustrates the dependence of enzyme velocity on dTDP-β-glucose concentrations at various concentrations of PP_i. Fig. 5B illustrates the same experiment with dUDP-β-glucose as a substrate. From these observations and others

\[ \begin{align*}
\text{Fig. 3.} & \quad \text{Effect of nucleotide triphosphates on the pyrophosphorylation of UDP-β-glucose. Conditions as in Fig. 2 with double the enzyme concentration. In A, } \bullet \text{ represents no inhibitor; } \square, 8 \times 10^{-3} \text{M dTTP; } \bigcirc, 1 \times 10^{-4} \text{M dTTP. In B, } \bullet \text{ represents no inhibitor; } \square, 4 \times 10^{-4} \text{M dUTP; } v \text{ represents micromoles of product formed per minute.}
\end{align*} \]

\[ \begin{align*}
\text{Fig. 4.} & \quad \text{Effect of nucleotide triphosphates on the pyrophosphorylation of dTDP-β-glucose as a function of PP_i concentration. Assay 1 was used. } \bullet, \text{ no additions; } \square, 3 \times 10^{-5} \text{M dTTP; } \triangle, 6 \times 10^{-5} \text{M dTTP; } \bigcirc, 5 \times 10^{-4} \text{M dUTP. The } K_m \text{ value for PP_i calculated from these data is } 4.3 \times 10^{-5} \text{M; the } K_i \text{ value for dTTP is } 7.2 \times 10^{-4} \text{M and for dUTP is } 7.7 \times 10^{-4} \text{M. } v \text{ represents micromoles of product formed per minute.}
\end{align*} \]

\[ \begin{align*}
\text{Fig. 5.} & \quad \text{Effect of substrate concentration on the dTDP-β-glucose pyrophosphorylase reaction. Assay 1 was used with the substrate concentration indicated. One-half the enzyme concentration was used in the experiment in B as in A. The insert in A is a replot of the reciprocal of the extrapolated maximal velocities as a function of the reciprocal of the substrate concentration. } v \text{ represents micromoles of product formed per minute.}
\end{align*} \]
TABLE III

Apparent Michaelis constants for substrates of dTDP-D-glucose pyrophosphorylase

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Additions</th>
<th>Apparent $K_m$ $M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>dTDP-D-glucose</td>
<td>Infinite PP$_1$</td>
<td>$3.6 \times 10^{-5}$</td>
</tr>
<tr>
<td>dUDP-D-glucose</td>
<td>Infinite PP$_1$</td>
<td>$8 \times 10^{-5}$</td>
</tr>
<tr>
<td>UDP-D-glucose</td>
<td>Infinite PP$_1$</td>
<td>$6 \times 10^{-5}$</td>
</tr>
<tr>
<td>PP$_1$</td>
<td>Infinite dTDP-D-glucose</td>
<td>$4.4 \times 10^{-5}$</td>
</tr>
<tr>
<td>PP$_1$</td>
<td>Infinite UDP-D-glucose</td>
<td>$9.0 \times 10^{-5}$</td>
</tr>
<tr>
<td>dTTP</td>
<td>Infinite UDP-D-glucose</td>
<td>$6.6 \times 10^{-4}$</td>
</tr>
<tr>
<td>dUTP</td>
<td>Infinite UDP-D-glucose</td>
<td>$1.4 \times 10^{-4}$</td>
</tr>
<tr>
<td>UDP-D-glucose-1-P</td>
<td>$1 \times 10^{-4} M$ $\alpha$-glucose-1-P</td>
<td>$2 \times 10^{-4}$ to $4 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\alpha$-D-Glucose-1-P</td>
<td>$1 \times 10^{-4} M$ $\alpha$-glucose-1-P</td>
<td>$2 \times 10^{-4}$</td>
</tr>
<tr>
<td>UDP-D-glucose-1-P</td>
<td>$1 \times 10^{-4} M$ $\alpha$-glucose-1-P</td>
<td>$1.3 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\alpha$-D-Glucose-1-P</td>
<td>$2 \times 10^{-5} M$ dTTP</td>
<td>$5 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\alpha$-D-Glucose-1-P</td>
<td>$2 \times 10^{-4} M$ dUTP</td>
<td>$1 \times 10^{-4}$</td>
</tr>
<tr>
<td>$\alpha$-D-Glucose-1-P</td>
<td>$5 \times 10^{-4} M$ UTP</td>
<td>$1 \times 10^{-5}$</td>
</tr>
</tbody>
</table>

Fig. 6. Inhibition of dUDP-D-glucose synthesis by UDP-D-glucose and dTDP-D-glucose. Assay 2 was used. The concentration of glucose-1-P was $1 \times 10^{-4}$ M. •, no addition; □, $1 \times 10^{-2} M$ UDP-D-glucose; ○, $6 \times 10^{-4} M$ dTDP-D-glucose; $\triangle$, represents micromoles formed per 3 minutes.

which are not illustrated, the apparent Michaelis constants for the sugar nucleotides and PP$_1$ were calculated and are summarized in Table III. The kinetic results with dTDP-D-glucose are quite different from those with dUDP-D-glucose (or UDP-D-glucose). The extrapolated maximal velocity is about 4 times higher for dUDP-D-glucose and UDP-D-glucose than with dTDP-D-glucose. The parallel lines seen in Fig. 5A are usually interpreted as indicative of a transaminase type mechanism (14); however, the dTDP-D-glucose pyrophosphorylase does not catalyze an exchange between $\alpha$-D-glucose-$^{14}$C-1-P and dTDP-D-glucose. No simple explanation is at present available for the peculiar kinetics of this enzyme. In addition, there is evidence that PP$_1$ (see below) can also bind to a site other than the active site.

In the direction of nucleotide diphosphate sugar synthesis the enzyme will utilize UTP or dUTP as well as dTTP. The reaction is inhibited by dTDP-D-glucose and UDP-D-glucose, and this inhibition is competitive with respect to the nucleotide triphosphate. This is illustrated in Fig. 6 with dUTP as a substrate. Similar data have been obtained with dTTP and UTP as substrates.

The apparent Michaelis constants for TTP, dUTP, and UTP calculated from Fig. 7 are $4 \times 10^{-6} M$, $2 \times 10^{-4} M$, and $1.3 \times 10^{-3} M$, respectively. However it should be noted that the extrapolated maximal velocities are nearly 5 times higher with UTP and dUTP than with TTP.

The purified enzyme will still catalyze the synthesis of dTDP-D-glucosamine (15), and this reaction is inhibited by dTDP-D-rhamnose, suggesting very strongly that both activities are a property of the same enzyme.

Fig. 7. The effect of nucleotide triphosphate concentration on reaction velocity. Assay 2 was used with $1 \times 10^{-4} M$ glucose-1-P. In A, • represents dUTP; □, UTP. Assays with TTP were carried out for 1 minute with 0.4 unit of enzyme. Assays in B were carried out for 3 minutes with 1.2 units of enzyme; $\upsilon$ represents (micromoles of product formed per minute per 0.4 unit of enzyme) $\times 10^3$. 

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Inhibition by dTDP-L-rhamnose—Figs. 8 to 12 illustrate the effect of TDP-L-rhamnose on enzyme activity. These observations can be summarized as follows. While the inhibition by TDP-L-rhamnose is reversed by increasing substrate concentration, the inhibition is not of a simple competitive type. This is shown for dTDP-n-glucose in Figs. 8 and 9, and for dTTP in Fig. 10. Fig. 11 shows that this inhibition can also be partially reversed by very high concentrations of PPi. The shape of the curves in Fig. 11 suggests that, at high concentrations, PPi can also bind to a site other than the active site. Whether this second PPi binding site is identical with the site to which TDP-P

\[ \text{FIG. 8. Inhibition of dTDP-n-glucose pyrophosphorolysis by dTDP-L-rhamnose. Assay 1 with the following additions of dTDP-L-rhamnose: } \bullet, \text{ no additions; } \bigtriangleup, 3 \times 10^{-5} \text{ M; } \Delta, 4 \times 10^{-5} \text{ M; } \square, 8 \times 10^{-5} \text{ M; } \nu \text{ represents micromoles of product formed per minute.} \]

\[ \text{FIG. 9 (left). Inhibition of dTDP-d-glucose pyrophosphorolysis by dTDP-L-rhamnose. } \bigcirc, 5 \times 10^{-4} \text{ M dTDP-d-glucose; } \blacktriangledown, 1 \times 10^{-4} \text{ M dTDP-D-glucose; } \blacktriangle, 2.5 \times 10^{-4} \text{ M dTDP-d-glucose; } \nu \text{ represents micromoles of product formed per minute.} \]

\[ \text{FIG. 10 (right). Effect of nucleotide triphosphate concentration on dTDP-L-rhamnose inhibition. Assay 2 was used with } 1 \times 10^{-4} \text{ M } \alpha\text{-glucose-1-P. } \bigtriangleup, 5 \times 10^{-5} \text{ M dUTP; } \blacktriangle, 1 \times 10^{-5} \text{ M dTTP; } \bigcirc, 2 \times 10^{-5} \text{ M dTTP; } \blacktriangledown, 1 \times 10^{-4} \text{ M dTTP.} \]

\[ \alpha\text{-Glucose-1-P does not behave like PPi in reversing the dTDP-L-rhamnose inhibition.} \]

The following compounds did not affect the activity of this enzyme when tested at concentrations of \( 1 \times 10^{-4} \text{ M: } \text{dTDP, dTDP-d-glucosamine, dTDP-d-mannose, and L-rhamnose.} \]

The data in Fig. 12 show that if the concentration of dTDP-L-rhamnose required to give 50% inhibition at the particular substrate concentration noted is plotted against the concentration of substrate nucleotide, one can extrapolate back to a \( K_I \) value of \( 2.2 \times 10^{-4} \text{ M} \) for dTDP-L-rhamnose which is independent of the substrate used.

The sigmoidal inhibition curves with TDP-L-rhamnose, similar to those obtained with many other feedback inhibitors (for review of some of these effects see Reference 16), can be ascribed to the interaction between several binding sites on the protein for the inhibitor.

The difference between the inhibition by product (strictly competitive) and the inhibition by TDP-L-rhamnose suggests that the inhibitor, as in other cases (16, 17), binds at a site other than the active site. This is supported by the heat inactivation data in Table II, in which it is shown that TDP-L-rhamnose does not protect the enzyme from heat inactivation, while other thymidine nucleotides which do bind to the active site do have a protective effect. dTDP-L-rhamnose (\( 7 \times 10^{-5} \text{ M} \)) does not reverse the protective effect of dTDP-n-glucose (\( 1 \times 10^{-4} \text{ M} \)) in the heat denaturation experiment. A second indication that dTTP and dTDP-n-glucose bind to the same site is the observation that, in the presence of dTTP, dTDP n-glucose is more effective in reversing the dTDP-L-rhamnose inhibition.

The observation that dTDP-L-rhamnose will not reverse the protection against heat denaturation of dTDP-n-glucose can be interpreted to indicate either that, in the presence of dTDP-L-rhamnose, substrate is bound in a way that is catalytically inactive, but still can protect the enzyme against denaturation, or that there are two binding sites for substrate thymidine nucleotides, the active site and a second site which is involved in the denaturation experiments.

\[ \text{FIG. 11 (left). Graph showing the effect of PPi on the inhibition of dTDP-N-glucose pyrophosphorolysis by dTDP-L-rhamnose. Assay 1 with the following additions of PPi: } \bullet, \text{ no additions; } \blacktriangle, 2 \times 10^{-7} \text{ M; } \square, 6 \times 10^{-7} \text{ M; } \nu \text{ represents micromoles of product formed per minute.} \]

\[ \text{FIG. 12 (right). Sigmoidal curves showing the effect of substrate concentration on the inhibition of dTDP-L-rhamnose. \( 1 \times 10^{-4} \text{ M } \alpha\text{-glucose-1-P.} \) } \bigtriangleup, 5 \times 10^{-5} \text{ M dUTP; } \blacktriangle, 1 \times 10^{-5} \text{ M dTTP; } \bigcirc, 2 \times 10^{-5} \text{ M dTTP; } \blacktriangledown, 1 \times 10^{-4} \text{ M dTTP.} \]
DISCUSSION

Feedback inhibition as a control has now been documented for many metabolic pathways (10). The particular example of this control mechanism described in the present communication provides a mechanism for the regulation of TDP-L-rhamnose biosynthesis, provided the cell has an independent mechanism for controlling the level of dTTP. This is necessary because of the reversal of dTDP-L-rhamnose inhibition by dTTP (Fig. 10). That the level of deoxyribonucleotides in the cell is subject to a variety of regulatory mechanisms has been documented for several cell types (e.g. see References 18–23).

The concentration of dTDP-L-rhamnose in the cell must be sufficiently high to function as a glycosyl donor in the synthesis of cell wall structures. In P. aeruginosa, Burger, Glaser, and Burton (10) have shown that for two rhamnosyltransferases involved in the sequential synthesis of a rhamnolipid the $K_m$ values for dTDP-L-rhamnose are $5.2 \times 10^{-2}$ M and $2 \times 10^{-4}$ M, respectively.

In Salmonella typhimurium, Nikaido and Nikaido (24) have reported that in the transfer of rhamnose from dTDP-L-rhamnose to the cell wall lipopolysaccharide, the $K_m$ value for the nucleotide was $2 \times 10^{-4}$ M. There Michaelis constants should be compared with the data in Fig. 11 that show that at a TTP concentration of $1 \times 10^{-5}$ M, the enzyme is 90% inhibited by $6 \times 10^{-5}$ M dTDP-L-rhamnose.

Many organisms do not contain significant levels of dTDP-L-rhamnose. In other organisms, for example in strains of Escherichia coli and Lactobacillus acidophilus (25), the dTDP-L-rhamnose concentration is quite high. In particular, Okazaki, Strominger, and Okazaki (26) have recently examined rough and smooth variants of a strain of S. welasco and E. coli 018. The smooth variants contain rhamnose in the cell wall, whereas the rough variants do not.

The acid-soluble nucleotide pool of rough variant of S. welasco contains no measurable concentration of dTDP-L-rhamnose, although the enzymes for its synthesis are still present in this organism. On the other hand, the rough strain of E. coli 018 contains high levels (24 μmoles/270 g of cell paste) of dTDP-L-rhamnose. It would be predicted therefore that the E. coli 018 dTDP-D-glucose pyrophosphorylase is not subject to inhibition by dTDP-L-rhamnose. We have tested the dTDP-glucose pyrophosphorylase in crude sonic extracts of E. coli 018 (ATCC 12743) and found that it was not inhibited by $1 \times 10^{-4}$ M dTDP-L-rhamnose (Assay 1). P. aeruginosa enzyme, added to the E. coli extract, was completely inhibited under these conditions.

Since the dTDP-L-rhamnose in the rough strain of E. coli 018 is considerably less than the rhamnose that the smooth form would have in its cell wall, it is clear that other control mechanisms must come into play under these conditions.

Many antibiotics will inhibit bacterial cell wall synthesis, and it has been consistently observed that only some microorganisms will accumulate nucleotide-linked cell wall precursor (for review see Reference 27). It is quite possible, in view of the observations presented in this paper, that only organisms which lack a feedback control mechanism will accumulate nucleotide cell wall precursors and, furthermore, that different nucleotide diphosphate derivatives may accumulate in different organisms, although the primary site of inhibition is the same.

The kinetics of the dTDP-D-glucose pyrophosphorylase from P. aeruginosa is obviously very complex and will require studies with a pure protein with which it should be possible not only to carry out direct binding studies but also to detect changes in the protein conformation, by other than kinetic methods. Any attempt to explain in detail the mechanism of the dTDP-D-glucose pyrophosphorylase must take into account the following observations. (a) Thymidine nucleotide substrates protect the enzyme against thermal denaturation; uridine and deoxyuridine nucleotide substrates and dTDP-L-rhamnose cannot...
A method for the preparation of a 200-fold purified deoxothyminediphosphate sugar pyrophosphorylase has been described. The enzyme has been shown to utilize the following nucleotide substrates with the apparent Michaelis constants indicated: deoxothyminediphosphate (4 × 10⁻⁴ M), deoxyuridinediphosphate (2 × 10⁻⁴ M), uridine diphosphate (1.3 × 10⁻⁴ M), uridine monophosphate (6 × 10⁻⁴ M), deoxyuridinediphosphate n-glucose (8 × 10⁻⁴ M), and deoxothyminediphosphate n-glucose (3 × 10⁻⁵ M).

The enzyme is inhibited by dTDP-L-rhamnose. This inhibitor binds to a site other than the active site and evidence has been presented that more than one inhibitor binding site is present on the enzyme molecule. The inhibition by dTDP-L-rhamnose is reversed by the addition of nucleotide substrates, and also by the addition of PPi. Some of the implications of these findings for the control of dTDP-L-rhamnose biosynthesis in Pseudomonas aeruginosa have been presented.

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The Nucleotide Specificity and Feedback Control of Thymidine Diphosphate
\(d\)-Glucose Pyrophosphorylase

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