Purification and characterization of TDP-D-glucose 4,6-dehydratase from anthracycline-producing streptomycetes

MARK W. THOMPSON,1,2† WILLIAM R. STROHL2* and HEINZ G. FLOSS1

1Department of Chemistry, University of Washington, Seattle, WA 98195, USA
2Department of Microbiology, The Ohio State University, Columbus, OH 43210, USA

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TDP-D-glucose 4,6-dehydratase, which converts TDP-D-glucose to TDP-D-4-keto-6-deoxyglucose, was purified to near-homogeneity from the daunorubicin and baumycin-producing organism Streptomyces sp. C5 (968-fold purification with a 41% recovery), and from the daunorubicin producer Streptomyces peucetius ATCC 29050 (1000-fold purification with a 37% recovery). The TDP-D-glucose 4,6-dehydratases from Streptomyces sp. C5 and S. peucetius were determined by SDS-PAGE and HPLC gel filtration to be homodimers with subunit relative molecular masses of 39000 and 36000, respectively. For the enzymes from both organisms, negligible activity was observed in the absence of added NAD+, or when ADP-glucose, ADP-mannose, GDP-mannose, UDP-glucose or UDP-galactose was substituted for TDP-D-glucose as substrate. For the enzyme from Streptomyces sp. C5, the $K_m$ values for NAD+ and TDP-D-glucose were 19.2 $\mu$M and 31.3 $\mu$M, respectively. The $V_{max}$ for TDP-D-glucose was 309 nmol min$^{-1}$ (mg protein)$^{-1}$. For the S. peucetius enzyme, the $K_m$ values for NAD+ and TDP-D-glucose were 20.1 $\mu$M and 34.7 $\mu$M, respectively. $V_{max}$ values were 180 nmol min$^{-1}$ (mg protein)$^{-1}$ for NAD+ and 201 nmol min$^{-1}$ (mg protein)$^{-1}$ for TDP-D-glucose. TDP was a good inhibitor of TDP-D-glucose 4,6-dehydratase from both organisms. The N-terminal amino acid sequence of the TDP-D-glucose 4,6-dehydratase from S. peucetius and from the erythromycin producer, Saccharopolyspora erythraea, were similar, whereas the enzyme from Streptomyces sp. C5 contained a different N-terminal amino acid sequence from either of the other two enzymes.

Introduction

Many antibiotics, including macrolides such as tylosin (Matern et al., 1973), erythromycin (Vara & Hutchinson, 1988), and the avermectins (Schulman et al., 1990), the benzoisochromane quinone, granaticin (Snipes et al., 1979), and anthracyclines such as daunorubicin (daunomycin), doxorubicin (adriamycin), and the aclacino-lyses (Glaser, 1969, 1973), is apparently the branch point between normal hexose metabolism and the formation of deoxyhexoses such as L-rhamnose (Glaser & Kornfeld, 1961). The enzymes have been purified from a variety of sources, including Escherichia coli (Wang & Gabriel, 1969; Zarkowsky & Glaser, 1969), Pasteurella pseudotuberculosis (Gonzalez-Porque & Strominger, 1972), Phaseolus vulgaris (Liao & Barber, 1972), and porcine thyroid tissue (Broschet et al., 1985). In the synthesis of glycosyl moieties of antibiotics, thymidine 5’-diphosphate (TDP) glucose 4,6-dehydratase (TDPGDH; EC 4.2.1.46) catalyses the conversion of TDP-D-glucose to TDP-D-4-keto-6-deoxyglucose (Matern et al., 1973; Grisebach, 1978; Snipes et al., 1979; Vara & Hutchinson, 1988). Glucose provides the carbon atoms for 2,6-dideoxyhexoses and other unique hexoses in antibiotics, and the transformations proceed without rearrangement of the carbon skeleton (Grisebach, 1978). The conversion of hexose nucleotides into 4-keto-6-deoxyhexose nucleotides, catalysed by nucleoside hexose 4,6-dehydratases (Gabriel, 1973), is apparently the branch point between normal hexose metabolism and the formation of deoxyhexoses such as L-rhamnose (Glaser & Kornfeld, 1961). The enzymes commonly lack functionalities at C-2 and C-6, but little detail is known about their mode of formation (Grisebach, 1978; Schulman et al., 1990; Snipes et al., 1979; Vara & Hutchinson, 1988).
be detected and partially purified from a tylosin-producer, *Streptomyces rimosus* (Matern et al., 1973), and its activity has been correlated with antibiotic biosynthesis in the chlorothricin-producer *S. antibioticus* Tü99, the granaticin producer *S. violaceoruber* Tü22 and the daunorubicin-producer *Streptomyces peucetius* ATCC 29050 (K. Goeke & H. G. Floss, unpublished results). Recently, TDPGDH was purified to homogeneity from the erythromycin-producing organism, *Saccharopolyspora erythraea* (formerly *Streptomyces erythreus*) (Vara & Hutchinson, 1988).

Doxorubicin, and its 14-deoxy analogue, daunorubicin, are the major anthracyclines used clinically in the United States for the treatment of neoplasias including breast, bladder, lung, ovarian, and thyroid cancers, osteogenic sarcoma, neuroblastomas, Hodgkin's disease and other lymphomas, and acute leukaemias (Crooke, 1981). The 2,3,6-trideoxy-3-amino hexose, daunosamine (Fig. 1), at C-7 of the anthracyclinone moiety is required for the antineoplastic activity of doxorubicin and daunorubicin (Fujiwara & Hoshino, 1983). Although little is known about the biosynthesis of TDP-daunosamine in anthracycline-producing streptomycetes, it seems likely that the first committed step would be the conversion of TDP-D-glucose to TDP-D-4-keto-6-deoxy-D-glucose catalysed by TDPGDH (Fig. 1). In this study, TDPGDH was purified to near-homogeneity from the baumycin and daunorubicin-producing strain *Streptomyces* sp. C5, and from the daunorubicin-producing strain *S. peucetius* ATCC 29050.

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Fig. 1. Abbreviated hypothetical pathway for the biosynthesis of daunorubicin from TDP-D-glucose, acetyl-CoA, and propionyl-CoA. Aglycone formation and the predicted glycosylation step for this hypothetical pathway are described in greater detail by Strohl et al. (1989) and Bartel et al. (1990).
Methods

Organisms and fermentation conditions. Streptomyces sp. C5 (Bartel et al., 1990; Strohl et al., 1989) was from the Frederick Cancer Research Center, Frederick, MD, and Streptomyces peucetius (Dekleva et al., 1985; Strohl et al., 1989) was obtained as ATCC 29050 from the American Type Culture Collection, Rockville, MD.

Spores scraped from plates of R2YE agar medium (Hopwood et al., 1985) were used to inoculate eight 250 ml baffled culture flasks containing seed medium, which contained (per litre): malt extract, 10 g; glucose, 10 g; peptone, 5 g; and yeast extract, 4 g (pH 7.0, adjusted with NaOH before autoclaving). Seed cultures were incubated for 3 d with rotary shaking (250 r.p.m.) at 30 °C and then were used to inoculate (inoculum size, 9–10%, v/v) stirred-tank fermenters containing NDYE medium, which consisted of (per litre): glucose, 22.5 g; yeast extract, 5 g; NaNO₃, 4.28 g; 3-(4-morpholino)propane sulfonate (MOPS), 4.18 g; KH₂PO₄, 0.174 g; and 10× trace element solution (Dekleva & Strohl, 1987). 2 ml. In S. peucetius fermentations, maltose was substituted for glucose in both seed and production media to prevent acidogenesis (Dekleva & Strohl, 1987).

Preliminary fermentations, in which TDPGDH activity was determined as a function of time, biomass accumulation and antibiotic production, were run for 5 d using fermenters (14 l, 101 working volume) as previously described (Dekleva & Strohl, 1988). Dry weights and total anthracycline production were analysed as described by Dekleva et al. (1985).

For purification of TDPGDH, cultures were grown in a (24 l, 201 working volume) New Brunswick CMF 128S fermenter under the following conditions: air flow, 10 l min⁻¹; agitation, 250 r.p.m.; temperature, 30 °C; the pH, initially 7.0, was not controlled. Streptomyces sp. C5 mycelia were harvested after 36 h of growth using a Pellicon tangential flow filtration apparatus (Millipore) followed by centrifugation at 39,000g for 20 min. 5% (w/v) glycerol, 0.1 M-EDTA, and protease inhibitors [1 mM-phenylmethylsulphonyl fluoride (PMSF) and 0.1 m-]leupeptin, and 0.1 mM-phenylmethylsulphonyl fluoride (PMSF)]. For purification of TDPGDH from S. peucetius, 98 g frozen mycelial paste was used.

All enzyme purification procedures were carried out at 4 °C and all buffers, including those used for equilibration and elution, contained 5% (w/v) glycerol, 5 mM-EDTA, and 1 mM-EDTA. Unbroken mycelia and cell walls were pelleted by centrifugation at 39,000g for 20 min. DNA and some proteins were precipitated from the supernatant by addition of streptomycin sulphate to a final concentration of 1% (w/v). After additional centrifugation at 39,000g as above, the supernatant was chromatographed on a DEAE-Sepharose column (10 x 5 cm) which had been equilibrated with 0.05 M-Tris/HC1 buffer (pH 7.8) containing 5 mM-dithionooreitol (DTT), 5% (w/v) glycerol, 1 mM-EDTA, and protease inhibitors [1 mM-pepstatin A, 0.1 mM-leupeptin, and 0.1 mM-phenylmethylsulphonyl fluoride (PMSF)]. For purification of TDPGDH from S. peucetius, 98 g frozen mycelial paste was used.

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Polyacrylamide gel electrophoresis. SDS-PAGE used a 12% (w/v) resolving gel and a 3% (w/v) stacking gel (O’Farrell, 1975). The final concentration of SDS in both gels was 0.1%. Samples were prepared by mixing 15–20 μg of protein in a buffer containing 60 mM-Tris/HC1 (pH 6.8), 5% (v/v) β-mercaptoethanol, 10% (w/v) glycerol, 0.1% SDS, and a few crystals of bromophenol blue. Electrophoresis was run at 15–20 mA per gel. Protein in the gels was detected using silver nitrate as described by Merrill et al. (1983).

Measurement of TDPGDH activity. TDPGDH activity was measured spectrophotometrically in a 107.5 μl enzyme reaction mixture containing 50 mM-Tris/HC1 (pH 7.5), 1 mM-NAD⁺, 1 mM-TDP-glucose, and approx. 0.03 to 0.06 units TDPGDH. The reaction mixture was incubated at 37 °C for 1 h, after which 75 μl of 100 mM-NaOH was added. This mixture was boiled for 15 min, after which A₂₅₀ was recorded using a Bausch and Lomb Spectronic 2000. The molar absorption coefficient at 320 nm used to calculate the amount of TDP-4-keto-6-deoxyglucose formed was 4500 mol⁻¹ cm⁻¹ (Masuii & Strominger, 1966). It should be noted that Wang & Gabriel (1969) used 4800 mol⁻¹ cm⁻¹, and Vara & Hutchinson (1988) and Zarkjowsky & Glaser (1969), 6500 mol⁻¹ cm⁻¹ as the molar absorption coefficients for this reaction. One unit of enzyme activity is defined as the amount of enzyme required to form 1 nmol TDP-4-keto-6-deoxyglucose per min under the described assay conditions. Protein was measured by the dye binding assay of Bradford (1976) using reagent purchased from BioRad.

Analysis of the enzyme reaction product. The product from the reaction between TDP-[U-¹⁴C]D-glucose (2 μCi; 0.6 mm) and NAD⁺, catalysed by purified TDPGDH, was separated from the reactant by thin-layer chromatography (TLC) on cellulose plates (Brinkman MN-300) using isobutyric acid/1 M-ammonium hydroxide (1:0:0-6) as the developing solvent. Radioactive nucleotide hexoses were detected by autoradiography using XAR-5 (Kodak) X-ray film, scraped from the plates into 50 μl ddH₂O, and reduced by treatment for 2 h with 50 μl NaBH₄ (10 mg ml⁻¹ in 20 mM-NaOH). The sugar nucleotides were then hydrolysed for 4 h at 37 °C with 9 μl of snake venom phosphodiesterase and 0.35 U of alkaline phosphatase in 0.05 M-Tris/HC1 (pH 9.4). Protein was precipitated from the mixture by heating for 5 min at 90 °C and removed by centrifugation. The solution containing the hexose products was lyophilized, resuspended in 20 ml ddH₂O, and chromatographed (TLC) on silica gel with chloroform/methanol (6:4) as the mobile phase. Hexoses were detected by silver staining (Trevelyan et al., 1950). Non-radioactive reaction products separated by cellulose TLC were also examined by fast atom bombardment mass spectrometry (FAB-MS) using a VG-70-2050 FAB mass spectrometer.

Kinetics and substrate and inhibitor specificity of TDPGDH. KΜ and VΜₘₐₓ were determined in triplicate for NAD⁺ and TDP-glucose by Lineweaver–Burk double reciprocal plots of enzyme reactions with a range of NAD⁺ and TDP-glucose concentrations. For the kinetics as a function of NAD⁺ concentration, TDP-glucose was in excess.
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(1 mM) and the concentration of NAD⁺ varied from 10 to 250 μM. For the kinetics as a function of TDP-D-glucose concentration, NAD⁺ was in excess (1 mM) and the concentration of TDP-D-glucose varied from 25 to 250 μM. The kinetic constants for NAD⁺ and TDP-D-glucose were calculated using the enzyme kinetic model fitting software EZ-FIT (Perella Scientific). This software program fits experimental data to enzyme kinetic models using the Nelder-Mead Simplex and Marquardt nonlinear regression methods.

Inhibition reactions were carried out in triplicate using 2 mM-inhibitor (TTP, TDP, or TMP). Alternative substrates (ADP-glucose, ADP-mannose, CDP-glucose, GDP-glucose, GDP-mannose, UDP-glucose, or UDP-galactose) were used at 1 mM.

N-terminal sequencing of TDPGDH. Purified TDPGDH was adsorbed on a pre-wetted polyvinylidene difluoride (PVDF) membrane during overnight incubation in distilled water with excised SDS-PAGE gel plugs. The N-terminal amino acid sequences of the purified enzyme from Streptomyces sp. C5 (16 pmol), and of the protein bands (15 pmol each) from S. peucetius thought to be the monomeric subunits of TDPGDH, were determined by Edman degradation using an automated gas-phase protein sequencer (Model 470A; Applied Biosystems) and an on-line phenylthiohydantoin (PTH) analyser (Model 120A; Applied Biosystems).

Reproducibility of results. Unless otherwise stated, all experiments were carried out at least in triplicate and the results given are the averages of those data. For purifications, all steps except the final gel filtration by HPLC (which was performed only once) were performed at least four times and the data given are for representative runs.

Results and Discussion

Fermentation of Streptomyces sp. C5 and S. peucetius

In Streptomyces sp. C5 fermentations, TDPGDH specific activity peaked between 24 and 42 h. The maximum rate of anthracycline production by Streptomyces sp. C5 was between 24 and 48 h (data not shown). On the other hand, in S. peucetius fermentations, the specific activity of TDPGDH remained constant between 48 and 120 h, during which period anthracyclines were continuously produced (data not shown). Thus, in both cases, the specific activity of TDPGDH correlated with anthracycline production, supporting a role for this enzyme in the biosynthesis of the daunosamine moiety.

Purification of TDPGDH from Streptomyces sp. C5

Streptomycin sulphate precipitated 120% of the TDPGDH activity in crude extracts. In preliminary experiments, the total activity precipitated was often 2- to 3-fold that in the crude enzyme fraction, suggesting that either an inhibitor of TDPGDH or an enzyme consuming the TDPGDH product had been removed. TDPGDH activity was eluted from DEAE-Sepharose at 0.28–0.32 M-KCl (Fig. 2a) with a 6.5-fold purification (Table 1). Precipitation of the active fractions with ammonium sulphate and fractionation by gel-filtration chromatography gave a single peak (Fig. 2b) with an additional purification of approx. 6-fold (Table 1). TDPGDH was further purified by FPLC on Mono Q using a linear gradient of 0 to 1 M-KCl in 0.05 M-PPB (pH 7.8). The enzyme eluted in a single, sharp peak at
of enzyme activity but achieved a purification of approx. 74 000 (data not shown); the TDPGDH was 0.36 M-KCl (Fig. 2c). This step resulted in a 2.6-fold loss of the enzyme (17.45 min) indicated a retention time of the enzyme (17.45 min) indicated a $M_r$ of approx. 74000 (data not shown); the TDPGDH was purified 968-fold from the crude extract with a recovery of 37% (data not shown).

Purification of TDPGDH from $S$. peucetius ATCC 29050

The same purification protocol as described above was used to purify TDPGDH from $S$. peucetius cell extracts (data not shown). $S$. peucetius TDPGDH eluted from the HPLC gel-filtration column at a retention time of 17.6 min, giving a $M_r$ of 68000 for the native protein. After the HPLC gel-filtration step, the TDPGDH from $S$. peucetius had been purified 1000-fold over crude cell-free extract with a recovery of 37% (data not shown).

**N-terminal amino acid sequences of the purified enzyme subunits**

The sequence of the first 22 amino acids from the N-terminus of TDPGDH from $S$. peucetius sp. C5 was determined (Fig. 4). The N-terminal amino acid sequence was markedly different from that of TDPGDH in the erythromycin-producer $S$. erythraea (Fig. 4). The TDPGDH from $S$. peucetius was not purified to homogeneity; however, only a few major bands

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**Table 1. Scheme for purification of TDPGDH from $S$. peucetius sp. C5**

All steps were carried out at least four times except the final TSK-3000 step which was done once. The data shown are from a representative purification run.

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Protein (mg)</th>
<th>Total activity (nmol min$^{-1}$)</th>
<th>Specific activity (nmol min$^{-1}$ (mg protein)$^{-1}$)</th>
<th>Purification factor</th>
<th>Recovery (%)</th>
</tr>
</thead>
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<tr>
<td>Crude extract</td>
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<td>270</td>
<td>0.31</td>
<td>1.0</td>
<td>100</td>
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<tr>
<td>Streptomycin sulphate</td>
<td>624</td>
<td>324</td>
<td>0.52</td>
<td>1.7</td>
<td>120</td>
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<tr>
<td>DEAE-Sepharose</td>
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<td>280</td>
<td>3.46</td>
<td>11</td>
<td>104</td>
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<tr>
<td>Sephades G-200</td>
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<td>20.3</td>
<td>65</td>
<td>98</td>
</tr>
<tr>
<td>Mono Q FPLC</td>
<td>0.41</td>
<td>100</td>
<td>244</td>
<td>787</td>
<td>37</td>
</tr>
<tr>
<td>TSK-3000 HPLC</td>
<td>0.37</td>
<td>111</td>
<td>300</td>
<td>968</td>
<td>41</td>
</tr>
</tbody>
</table>

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Fig. 3. Determination of $M_r$ for the polypeptide subunits of the TDPGDH enzymes from $S$. peucetius sp. C5 and $S$. peucetius ATCC 29050 by SDS-PAGE analysis. Lanes 1 and 5, SDS-PAGE standards; bovine serum albumin (66000), egg albumin (45000), subunit of glyceraldehyde 3-phosphate dehydrogenase (36000), carbonic anhydrase (29000), trypsinogen (24000), trypsin inhibitor (20100), and $\alpha$-lactalbumin (14200). Lane 2, subunit of purified TDPGDH from $S$. peucetius sp. C5 after chromatography through the HPLC gel-filtration step, calculated to have a $M_r$ of 39000. Lane 3, peptides still present in preparation of TDPGDH from $S$. peucetius after chromatography through the HPLC gel-filtration step. Lane 4, peptide presumed to be the monomer of TDPGDH from $S$. peucetius which was eluted from the gel at the stage indicated in Lane 3 and sequenced (see Fig. 4). This peptide was calculated to have a $M_r$ of 36000. The data shown in lanes 4 and 5 are from separate gels; hence, the peptides migrated slightly differently from those shown in lanes 1-3.

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(a) NH$_2$-Asp-Glu-Leu-Phe-Tyr-Asp-Ala-Asp-Ala-Asp-Leu-Ser-Ile-Ile-Gln-Gly-Arg-Lys-Val-Ala-Val-Ile-

(b) NH$_2$-Thr-Xxx-Thr-Pro-Val-Asn-Val-Thr-Val-Thr-Gly-Ala-Ala-Gly-Ile-Gly-Ile-Gly-Tyr-Ala-Leu-

(c) Xxx-Arg-Val-Leu-Val-Thr-Gly-Gly-Ala-Ala-Gly-Phe-Ile-Gly-Ser-His-

Fig. 4. The N-terminal amino acid sequence of: (a) the peptide monomer of the purified TDPGDH from $S$. peucetius sp. C5; (b) the $M_r = 36000$ peptide from $S$. peucetius ATCC 29050 believed to be the monomer of TDPGDH from that organism; and (c) the TDPGDH from $S$. erythraea (Vara & Hutchinson, 1988) (unpublished sequence data courtesy of C. R. Hutchinson) (sequence C, bottom line). The bottom two sequences are aligned for maximum conservation; conserved amino acids are indicated (□).
obtained by a purification protocol that had yielded a near-homogeneous enzyme from TDPGDH from Streptomyces sp. C5 (Fig. 3). Attempts to remove the contaminating proteins from the final S. peucetius preparation, including hydrophobic chromatography, isoelectric focussing and chromatofocussing, were not successful; therefore, the two major peptides in the 30000 to 40000 range [the most probable size based upon the properties of TDPGDH from Streptomyces sp. C5 and Sac. erythraea (Vara & Hutchinson, 1988) and the HPLC gel-filtration data on the S. peucetius enzyme] were excised from an SDS-PAGE gel and their N-terminal amino acids were sequenced. The polypeptide with 72000 and a subunit Mr of 36000 was a homodimer with a Mr of 72000 and a subunit Mr of 36000 (Table 2). The pHs for optimum activity of TDPGDH from Streptomyces sp. C5 and S. peucetius are 7-6 and 7-8, respectively (Table 2). The enzymes from S. rimosus (Matern et al., 1973) and Sac. erythraea (Vara & Hutchinson, 1988) had optima at pH 7-2 and 7-5, respectively, whereas E. coli TDPGDH had a pH optimum in the range of 8-0-8-5 (Wang & Gabriel, 1969; Zarkowsky & Glaser, 1969).

Table 2. Comparison of the characteristics of TDPGDH from Streptomyces sp. C5, S. peucetius, Sac. erythraea and E. coli

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Streptomyces C5</th>
<th>S. peucetius</th>
<th>Sac. erythraea</th>
<th>E. coli</th>
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<tr>
<td>$M_r$, of native enzyme</td>
<td>78000</td>
<td>72000</td>
<td>72000</td>
<td>78000</td>
</tr>
<tr>
<td>$M_r$, of subunits</td>
<td>39000</td>
<td>36000</td>
<td>36000</td>
<td>39000</td>
</tr>
<tr>
<td>Enzyme structure</td>
<td>Homodimer</td>
<td>Homodimer</td>
<td>Homodimer</td>
<td>Homodimer</td>
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<tr>
<td>Activity in absence of NAD$^+$</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Optimum pH for activity</td>
<td>7-6</td>
<td>7-8</td>
<td>7-5</td>
<td>8-0-8-5</td>
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<tr>
<td>Kinetics</td>
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<tr>
<td>$K_{m}$ (TDP-D-glucose)</td>
<td>31 $\mu$M</td>
<td>35 $\mu$M</td>
<td>34 $\mu$M</td>
<td>70 $\mu$M</td>
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<tr>
<td>$V_{max}$ (TDP-D-glucose)</td>
<td>309</td>
<td>201</td>
<td>433</td>
<td>7000</td>
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<tr>
<td>$K_{m}$ (NAD$^+$)</td>
<td>19 $\mu$M</td>
<td>20 $\mu$M</td>
<td>19 $\mu$M</td>
<td>100-200 $\mu$M</td>
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<td>Inhibition by*</td>
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<tr>
<td>TTP (2 mM)</td>
<td>37%</td>
<td>40%</td>
<td>77%</td>
<td>ND</td>
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<td>58%</td>
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<td>TMP (2 mM)</td>
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<td>23%</td>
<td>11%</td>
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<td>This work</td>
<td>This work</td>
<td>a</td>
<td>b</td>
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</table>

ND, not determined by cited authors.

−, Catalytic amounts of NAD$^+$ required for activity.

+ , NAD$^+$ tightly bound to enzyme, removable only with thiol reagents (Wang & Gabriel, 1969) or by acid denaturation (Vara & Hutchinson, 1988).

* These data are averages of three determinations. For these experiments, the control (100%) activities for the enzymes from Streptomyces sp. C5 and S. peucetius were 277 and 171 nmol min$^{-1}$ (mg protein)$^{-1}$, respectively.


Characterization of the optimal enzyme reaction conditions

The pHs for optimum activity of TDPGDH from Streptomyces sp. C5 and S. peucetius are 7-6 and 7-8, respectively (Table 2). The enzymes from S. rimosus (Matern et al., 1973) and Sac. erythraea (Vara & Hutchinson, 1988) had optima at pH 7-2 and 7-5, respectively, whereas E. coli TDPGDH had a pH optimum in the range of 8-0-8-5 (Wang & Gabriel, 1969; Zarkowsky & Glaser, 1969).

Neither the Streptomyces sp. C5 nor the S. peucetius TDPGDH was active when either ADP-glucose, ADP-mannose, GDP-glucose, GDP-mannose, UDP-glucose, or UDP-galactose was substituted for TDP-D-glucose (Table 2). The E. coli enzyme also did not utilize UDP-glucose as an alternative substrate but was capable of using dUDP-d-glucose, albeit with a substantially lower affinity (Zarkowsky & Glaser, 1969).

The enzymes from both streptomycetes gave a linear increase in absorbance for 60 min in the presence of excess TDP-D-glucose and NAD$^+$ (data not shown). Very low enzyme activity (i.e. less than 10% of maximum) was observed for TDPGDH from both...
sources in assay mixtures lacking NAD\(^+\); a similar NAD\(^+\) requirement was reported for TDPGDH from Sac. erythraea (Vara & Hutchinson, 1988), S. rimosus (Matern et al., 1973), and Pseudomonas aeruginosa (Glaser & Kornfeld, 1961), but in contrast, TDPGDH from E. coli strain B has tightly bound NAD\(^+\) (Wang & Gabriel, 1969; Zarkowsky & Glaser, 1969).

Identification of the TDPGDH reaction product of Streptomyces sp. C5

The product of the reaction catalysed by TDPGDH was analysed by TLC, reduction and enzymic cleavage to detect the predicted hexoses, and by FAB-MS to detect the expected product eluted from the TLC plates was also reduced and hydrolysed with snake venom phosphodiesterase, the two products co-migrated on silica gel TLC plates with authentic 6-deoxy-6-galactose) (data not shown), which are the expected M\(+\) for TDP-D-glucose of TDPGDH from S. C5 and S. peucetius, the product formed had the same R\(_f\) value as the product of the Streptomyces sp. C5 enzymic reaction.

Kinetics of TDPGDH activity from Streptomyces sp. C5 and S. peucetius

\(K'_m\) and \(V'_{max}\) values for TDPGDH from Streptomyces sp. C5 and S. peucetius are shown in Table 2. The \(K'_m\) values for TDP-D-glucose of TDPGDH from S. peucetius, Streptomyces sp. C5 and Sac. erythraea are all approx. 31-35 \(\mu\)M, whereas for the E. coli enzyme the \(K'_m\) is 70 \(\mu\)M (Wang & Gabriel, 1969; Zarkowsky & Glaser, 1969). The partially purified TDPGDH from the tylosin-producing strain, S. rimosus, had a \(K'_m\) for TDP-D-glucose of 93 \(\mu\)M (Matern et al., 1973). With NAD\(^+\) as substrate, the \(K'_m\) values for the streptomycete enzymes were 19-20 \(\mu\)M, a range approx. 5-10 fold lower than for the E. coli enzyme (Table 2).

Inhibition of TDPGDH by nucleotides

Inhibition was determined by including thymidine 5'-triphosphate (TTP), TDP, or thymidine 5'-monophosphate (TMP), and TDP-D-glucose in enzyme mixtures. TDP had the greatest effect, inhibiting enzyme activity from both organisms (Table 2). With 2 mM-TDP, Streptomyces sp. C5 and S. peucetius TDPGDH activities were only 38% and 42% of the value for the control.

Involvement of TDPGDH in daunosamine biosynthesis

The enzymes from Streptomyces sp. C5 and S. peucetius were optimally active during peak anthracycline production, not during exponential growth as would be expected for enzymes of primary metabolism (Dekleva & Strohl, 1988). This suggests that these enzymes participate in daunosamine biosynthesis rather than in the synthesis of a growth constituent such as the cell wall. Moreover, only a single TDPGDH activity was detected in the extracts of each organism (M. W. Thompson, unpublished results). Interestingly, the specific activities of TDPGDH in crude extracts of both Streptomyces sp. C5 and S. peucetius were in the same range as the specific activity of aklanonic acid methyltransferase, an enzyme of anthracyclinone formation (N. C. Connors, M. Dickens & W. R. Strohl, unpublished data). In vitro reactions in which mycelial extract of Streptomyces sp. C5 was incubated for 3 h at 37 °C with a reaction mixture containing 48 mM-Tris/HCl (pH 7.6), TDP-[U-\(^{14}\)C]glucose (2 \(\mu\)Ci; 0.6 mM), 0.6 mM-NAD\(^+\), 0.72 mM-NADPH, 3.4 mM-L-glutamate, and 3 \(\mu\)g pyridoxylamine phosphate ml\(^{-1}\) resulted in the formation of a TDP-sugar that, when reduced and hydrolysed with snake venom phosphodiesterase, co-migrated with authentic daunosamine (M. W. Thompson, unpublished results). This suggests that TDP-D-glucose can be converted to TDP-daunosamine by Streptomyces sp. C5 mycelial extracts, which further suggests that the TDP-sugar is the nucleotide form of sugar used in daunosamine formation. Studies are now being carried out to determine the possible genetic linkage of the genes encoding TDPGDH of S. peucetius and Streptomyces sp. C5 with genes encoding anthracycline formation.

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