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

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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 39 000 and 36 000, 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 μ M and 31.3 μ 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 μ M and 34.7 μ 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 aclacinomycins (Fujiwara & Hoshino, 1983) contain partially deoxygenated hexose sugar components that are usually essential for biological activity. Deoxygenated hexoses in antibiotics 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). 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 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). It has

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Abbreviations: TDPGDH, TDP-D-glucose 4,6-dehydratase; PPB, KH₂PO₄/K₂HPO₄ buffer; PVDF, polyvinylidene difluoride; PTH, phenylthiohydantoin; NYDE, nitrate defined yeast extract medium; ddH₂O, double-distilled water; K'_m , apparent K_m ; V'_{max} , apparent V_{max} .

sarcoma, neuroblastomas, Hodgkin's disease and other lymphomas, and acute leukaemias (Crooke, 1981). The 2,3,6-trideoxy-3-amino hexose, daunosamine (Fig. 1), a 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-deoxyglucose catalysed by TDPGDH (Fig. 1). In this study, TDPGDH was purified to near-homogeneity from the baumycins and daunorubicin-producing strain *Streptomyces* sp. C5, and from the daunorubicin-producing strain *S. peucetius* ATCC 29050.

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-(*N*-morpholino)propane sulphonate (MOPS), 4.18 g; K₂HPO₄, 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, 10 l 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, 20 l 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 16000 *g* for 30 min (4 °C). *S. peucetius* mycelia were harvested after 120 h of growth by centrifugation at 16000 *g* for 30 min (4 °C).

Purification of TDPGDH. *Streptomyces* sp. C5 (10 l of frozen mycelial paste) was disrupted with an automated French press (American Instrument Co.) at 15000–16000 p.s.i. in 200 ml 0.05 M-Tris/HCl buffer (pH 7.8) containing 5 mM-dithiothreitol (DTT), 5% (w/v) glycerol, 1 mM-EDTA, and protease inhibitors [1 mM-pepstatin A, 0.1 µM-leupeptin, and 0.1 µM-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-DTT, and 1 mM-EDTA. Unbroken mycelia and cell walls were pelleted by centrifugation at 39000 *g* 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 39000 *g* as above, the supernatant was chromatographed on a DEAE-Sephadex column (10 × 5 cm) which had been equilibrated with 0.05 M-Tris/HCl (pH 7.8). After sample application, the column was washed with 100 ml equilibration buffer; then the enzyme was eluted with a linear gradient of 0 to 0.5 M-KCl in 0.05 M-Tris/HCl buffer (pH 7.8). The enzyme-containing fractions were combined and concentrated by precipitation with ammonium sulphate (65% of saturation; 5 °C). The pellet was resuspended in 2 ml of 0.05 M-Tris/HCl (pH 7.8) containing 0.1 M-KCl and the soluble proteins were size-fractionated through a Sephadex G-200 gel-filtration column (150 × 1 cm) using 0.05 M-Tris/HCl (pH 7.8) containing 0.1 M-KCl as the elution buffer. The TDPGDH was then concentrated using a Centriprep 10 ultrafiltration system (Amicon) and exchanged into 0.05 M-KH₂PO₄-K₂HPO₄ buffer (pH 7.8; PPB). The enzyme was then loaded on a Mono Q column that

had been equilibrated with 0.05 M-PPB (pH 7.8), washed with equilibration buffer, and eluted from the column with a 60 ml linear gradient of 0 to 1.0 M-KCl in 0.05 M-PPB (pH 7.8) essentially as described by Vara & Hutchinson (1988). The flow rate was maintained at 2 ml min⁻¹ and protein peaks were detected by absorbance at 280 nm. TDPGDH obtained from the Mono Q column was concentrated using a Centriprep 10 as above and loaded on a Progel-TSK 3000SWXL (Supelco) gel-filtration HPLC column. The enzyme was separated from contaminating proteins by HPLC gel-filtration using a mobile phase of 0.05 M-PPB (pH 7.5) containing 0.1 M-KCl. An Altex model 100A HPLC and an Altex model 100-40 flow-through cell in a Hitachi spectrophotometer set to 280 nm were used for HPLC analyses. Chromatographic separations were recorded with a Hewlett-Packard model 3390A integrator.

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/HCl (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 enzymic reaction mixture containing 50 mM-Tris/HCl (pH 7.5), 1 mM-NAD⁺, 1 mM-TDP-D-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 incubated for 15 min, after which A₃₂₀ was recorded using a Bausch and Lomb Spectronic 2000. The molar absorption coefficient at 320 nm used to calculate the amount of TDP-D-4-keto-6-deoxyglucose formed was 4500 l mol⁻¹ cm⁻¹ (Matsushashi & Strominger, 1966). It should be noted that Wang & Gabriel (1969) used 4800 l mol⁻¹ cm⁻¹, and Vara & Hutchinson (1988) and Zarkowsky & Glaser (1969), 6500 l 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-D-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 0.14 U of snake venom phosphodiesterase and 0.35 U of alkaline phosphatase in 0.05 M-Tris/HCl (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 µl 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 spectroscopy (FAB-MS) using a VG-70-250S FAB mass spectrometer.

Kinetics and substrate and inhibitor specificity of TDPGDH. *K_m* and *V_{max}* were determined in triplicate for NAD⁺ and TDP-D-glucose from Lineweaver-Burk double reciprocal plots of enzyme reactions with a range of NAD⁺ and TDP-D-glucose concentrations. For the kinetics as a function of NAD⁺ concentration, TDP-D-glucose was in excess

(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 (10 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

Fig. 2. Chromatographic steps used in the purification of TDPGDH from *Streptomyces* sp. C5. For each step, protein (●) and enzyme activity (▲) were measured as described in Methods. (a) DEAE-Sepharose ion-exchange chromatography profile; the enzyme was eluted at 0.28 to 0.32 M-KCl. (b) Sepharose G-200 gel-filtration chromatography profile; the enzyme was eluted from the column at 99–129 ml. (c) Mono Q FPLC ion-exchange chromatography profile; the enzyme was eluted from the column at 0.36 M-KCl in phosphate buffer (PPB).

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 1.0 M-KCl in 0.05 M-PPB (pH 7.8). The enzyme eluted in a single, sharp peak at

Table 1. Scheme for purification of TDPGDH from *Streptomyces* 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.

Purification step	Protein (mg)	Total activity (nmol min ⁻¹)	Specific activity [nmol min ⁻¹ (mg protein) ⁻¹]	Purification factor	Recovery (%)
Crude extract	866	270	0.31	1.0	100
Streptomycin sulphate	624	324	0.52	1.7	120
DEAE-Sepharose	81	280	3.46	11	104
Sephadex G-200	13	264	20.3	65	98
Mono Q FPLC	0.41	100	244	787	37
TSK-3000 HPLC	0.37	111	300	968	41

0.36 M-KCl (Fig. 2c). This step resulted in a 2.6-fold loss of enzyme activity but achieved a purification of approx. 12-fold (Table 1). Pure TDPGDH was obtained by high-performance liquid chromatography (HPLC) on a Progel-TSK 3000SWXL gel-filtration column. The

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 41% recovery (Table 1). The M_r of the monomeric polypeptide subunit given by SDS-PAGE was 39000 (Fig. 3). Since SDS-PAGE analysis is more precise than gel-filtration, the native enzyme is presumed to be a homodimer of $M_r = 78000$ containing monomeric subunits of $M_r = 39000$.

Fig. 3. Determination of M_r for the polypeptide subunits of the TDPGDH enzymes from *Streptomyces* 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 α -lactalbumin (14200). Lane 2, subunit of purified TDPGDH from *Streptomyces* 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.

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

- (a) NH₂-Asp-Glu-Leu-Phe-Tyr-Asp-Ala-Asp-Ala-Asp-Leu-Ser-Ile-Ile-Gln-Gly-Arg-Lys-Val-Ala-Val-Ile-
- (b) NH₂-Thr-Xxx-Thr-Pro-Val-Asn-Val-Thr-Val-Thr-Gly-Ala-Ala-Gly-Gln-Ile-Gly-Tyr-Ala-Leu-
- (c) Xxx-Arg-Val-Leu-Val-Thr-Gly-Gly-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 *Streptomyces* 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 *Sac. 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 (■).

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

All experiments shown in this table were done in triplicate. The data shown are averages of the results obtained. In no cases were the data points more than 10% from the mean shown. K'_m and V'_{max} values are rounded off to integers. V'_{max} values are given in $\text{nmol min}^{-1} (\text{mg protein})^{-1}$. The V'_{max} value for the *S. peucetius* enzyme on TDP-D-glucose is shown in brackets since the enzyme was only one of five major proteins in the assay mixture. Although the percent TDPGDH of total protein was calculated by scanning densitometry of the gels (data not shown), the value given is only an estimate, considering the amount of contaminating protein (see Fig. 3, lane 3).

Characteristic	<i>Streptomyces</i> C5	<i>S. peucetius</i>	<i>Sac. erythraea</i>	<i>E. coli</i>
M_r of native enzyme	78000	72000	72000	78000
M_r of subunits	39000	36000	36000	39000
Enzyme structure	Homodimer	Homodimer	Homodimer	Homodimer
Activity in absence of NAD^+	—	—	—	+
Optimum pH for activity	7.6	7.8	7.5	8.0–8.5
Kinetics				
K'_m (TDP-D-glucose)	31 μM	35 μM	34 μM	70 μM
V'_{max} (TDP-D-glucose)	309	201	433	7000
K'_m (NAD^+)	19 μM	20 μM	19 μM	100–200 μM
Inhibition by*				
TTP (2 mM)	37%	40%	77%	ND
TDP (2 mM)	62%	58%	90%	ND
TMP (2 mM)	25%	23%	11%	ND
References†	This work	This work	a	b

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 $\text{nmol min}^{-1} (\text{mg protein})^{-1}$, respectively.

† References: a, Vara & Hutchinson, 1988; b, Wang & Gabriel, 1969, and Zarkowsky & Glaser, 1969.

remained in the SDS-PAGE gels of the product, obtained by a purification protocol that had yielded a near-homogeneous enzyme 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 M_r 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 M_r 36000 had an N-terminal sequence similar to that of TDPGDH from *Sac. erythraea* (Fig. 4). The sequence of the M_r = approx. 40000 peptide showed no similarity to those of the *Streptomyces* sp. C5 or *Sac. erythraea* TDPGDH (data not shown). From the combined HPLC gel-filtration data (which show that the *S. peucetius* enzyme is slightly smaller than that of *Streptomyces* sp. C5), the SDS-PAGE data, and the N-terminal amino acid sequences, the native enzyme appears to be a homodimer with a M_r of 72000 and a subunit M_r of 36000 (Table 2).

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, CDP-glucose, 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 TDP-D-keto-6-deoxyglucose. When TDP-[U- ^{14}C]glucose (251 mCi mmol $^{-1}$; ICN Pharmaceuticals) was incubated with NAD^+ and purified TDPGDH from *Streptomyces* sp. C5, the product formed migrated at an $R_{\text{TDP-D-glucose}}$ (i.e. mobility in relation to TDP-D-glucose) of 1.2 during TLC on cellulose. When this compound was eluted from the TLC plates and subjected to FAB-MS, it gave a molecular ion of 569, which is the expected $M+1$ for TDP-D-4-keto-6-deoxyglucose monosodium salt (data not shown). The reaction product eluted from the TLC plates was also reduced with NaBH_4 and enzymically hydrolysed; the two products co-migrated on silica gel TLC plates with authentic quinovose (6-deoxy-D-glucose) and fucose (6-deoxy-D-galactose) (data not shown), which are the expected products of the non-stereospecific reduction and hydrolysis of TDP-D-4-keto-6-deoxyglucose (Glaser & Kornfeld, 1961). The product formed by the partially purified TDPGDH from *S. peucetius* 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 μM , whereas for the *E. coli* enzyme the K'_m is 70 μM (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 μM (Matern *et al.*, 1973). With NAD^+ as substrate, the K'_m values for the streptomycete enzymes were 19–20 μM , 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 anthracycline 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 μCi ; 0.6 mM), 0.6 mM- NAD^+ , 0.72 mM-NADPH, 3.4 mM-L-glutamate, and 3 μg 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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