

Enzymatic Conversion of Glucose to UDP-4-Keto-6-Deoxyglucose in *Streptomyces* spp.

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All of the 2,6-dideoxy sugars contained within the structure of chromomycin A₃ are derived from D-glucose. Enzyme assays were used to confirm the presence of hexokinase, phosphoglucomutase, UDPG pyrophosphorylase (UDPGP), and UDPG oxidoreductase (UDPGO), all of which are involved in the pathway of glucose activation and conversion into 2,6-dideoxyhexoses during chromomycin biosynthesis. Levels of the four enzymes in *Streptomyces* spp. cell extracts were correlated with the production of chromomycins. The pathway of sugar activation in *Streptomyces* spp. involves glucose 6-phosphorylation by hexokinase, isomerization to G-1-P catalyzed by phosphoglucomutase, synthesis of UDPG catalyzed by UDPGP, and formation of UDP-4-keto-6-deoxyglucose by UDPGO.

Dideoxy sugars occur commonly in the structures of cardiac glycosides from plants, in antibiotics like chromomycin A₃ (Fig. 1), and in macrolides produced by microorganisms. On the basis of stable isotope-labeling experiments, biosynthetic studies conducted in Rosazza's laboratory have indicated that all the deoxy sugars of chromomycin A₃ are derived from D-glucose (21). While the assembly of the polyketide aglycone is reasonably well understood, relatively little is known of the details of 2,6-dideoxy sugar biogenesis in streptomycetes. Earlier studies with *Streptomyces rimosus* indicated that TDP-my-carose is synthesized from TDP-D-glucose (TDPG) and S-adenosyl-L-methionine (10, 23). The reaction requires NADPH as a cofactor, and TDP-4-keto-6-deoxy-D-glucose is an intermediate. Formation of TDP-4-keto-6-deoxy-D-glucose was catalyzed by the enzyme TDPG oxidoreductase (TDPG-4,6-dehydratase; EC 4.2.1.46). Similar 4-keto sugar nucleotides are intermediates for the biosynthesis of polyene macrolide antibiotic amino sugars (18). Similar pathways have been elaborated for the formation of 2,6-dideoxy-D-threo-4-hexulose of granaticin in *Escherichia coli* (6, 25) and 2,6-dideoxy-D-arabino-hexose of chlorothricin (12). The initial 6-deoxygenation of glucose during 3,6-dideoxy sugar formation involves a similar mechanism (32). In all of these processes, glucose is first activated by conversion into a sugar nucleotide such as UDPG followed by NAD⁺ oxidation of the 4 position to the corresponding 4-oxo derivative. Position 6 deoxygenation involves a general tautomerization, dehydration, and NADH,H⁺-catalyzed reduction process (6, 12, 25). A similar tautomerization and dehydration followed by reduction may produce C-3-deoxygenated products, such as CDP-3,6-dideoxyglucose (27). The pathway for formation of 3,6-dideoxyhexoses from CDPG in *Yersinia pseudotuberculosis* was clearly elucidated by Liu and Thorson (14). However, none of this elegant work was focused on the earlier steps of hexose nucleotide formation.

On the basis of previous work (7), it is reasonable to postu-

late that the biosynthesis of 2,6-dideoxyglucose in *Streptomyces griseus* involves phosphorylation to glucose-6-phosphate by hexokinase (HK; E.C.2.7.7.1), as in glycolysis; conversion to glucose-1-phosphate by phosphoglucomutase (PGM; EC 2.7.5.1); reaction with UTP to form UDPG in a reaction catalyzed by UDPG pyrophosphorylase (UDPGP) (glucose-1-phosphate uridylyltransferase; EC 2.7.7.9), and C-6 deoxygenation catalyzed by UDP-D-glucose-4,6-dehydratase with NAD⁺ as a cofactor (Fig. 2). UDPG and GDPG have been detected in cell extracts of *S. griseus* and *Streptomyces* sp. strain MRS202, suggesting that these compounds are active sugar nucleotides involved in the formation of dideoxyhexoses (15). UDPGP genes from several bacteria have been cloned and sequenced (1, 3, 4, 11, 29, 30). Although nucleotidyl diphosphohexose-4,6-dehydratases (NDP-hexose-4,6-dehydratases) have been purified and characterized from several sources (5, 8, 9, 13, 19, 25, 26, 31, 33), the occurrence of the glucose-activating enzymes HK, PGM, UDPGP, and UDPG oxidoreductase (UDPGO) involved in 2,6-dideoxyhexose formation has not been established in streptomycetes. This work provides evidence for the presence of these enzymes involved in the biosynthetic activation of glucose to the 2,6-dideoxyhexoses in chromomycin A₃.

MATERIALS AND METHODS

General material and instrumentation. High-performance liquid chromatography (HPLC) was performed with a Rheodyne injector type 7125 with a 100- μ l loop connected to a model LC-6A pump, an SPD-6AV module UV-VIS detector, a CR-501 Chromatopac recording integrator, and an SCL-6B system controller, all from Shimadzu Co. (Osaka, Japan). The analytical column (250- by 4.6-mm inside diameter) was packed with 5- μ m partisisil octyldecyl silane/C₁₈ (Whatman Inc., Clifton, N.J.) and preceded by a guard column of the same composition (Alltech Inc., Deerfield, Ill.). UV and visible light spectroscopy was performed with a Shimadzu UV-160 spectrophotometer. Low-resolution fast atom bombardment-mass spectrometry spectra were measured on a ZAB-HF instrument by triethanolamine as a matrix solvent recorded in the negative-ion mode, and also with a Trio-III instrument in the University of Iowa Core Mass Spectrometry Facility.

Centrifugations were conducted with a Sorvall RC-5 refrigerated centrifuge (GSA or SS 35 rotors), or a Beckman model L8-55 refrigerated ultracentrifuge (type 35 or type 40 rotors), or a bench top Eppendorf microcentrifuge. Cell disruption was performed with a French press (SLM Instruments, Urbana, Ill.). A lyophilizer (The Virtis Co., Inc., Gardiner, N.Y.) was used for all sample lyophilizations.

HPLC-grade KH₂PO₄ and H₃PO₄ (85%) were both from Fisher Scientific (Fair Lawn, N.J.). Acetonitrile (HPLC grade) was from E. M. Science (Gibbstown, N.J.) and was filtered through type HV 0.45- μ m-pore-size Millipore (Bedford, Mass.) membranes before use.

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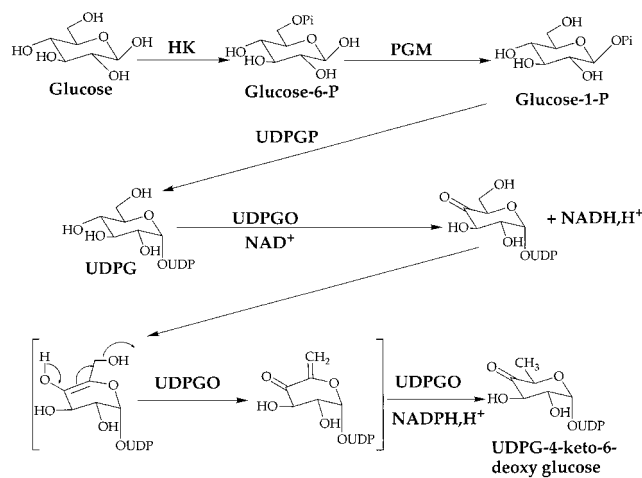


FIG. 2. Proposed pathway for the formation of 2,6-dideoxy sugars in streptomycetes involving HK, PGM, UDPGP, and UDPGO.

UDPG in the glucose activation pathway by incubating uniformly labeled [^{14}C]glucose with *Streptomyces* sp. strain MRS202. When cells were harvested from labeled glucose containing incubations, disrupted by French press homogenization, and analyzed by HPLC, a radioactive peak was eluted at a retention volume of 26 ml, consistent with the formation of [^{14}C]UDPG by *Streptomyces* sp. (15).

The formation of UDPG would typically involve the glucose activation pathway summarized in Fig. 2. The enzymes required include HK, PGM, UDPGP, and UDPGO. Assays were established in order to detect these enzyme activities in cell extracts of the *Streptomyces* species in this work. Results have been expressed in specific enzyme activities in order to correct for subtle differences in cell breakage and recovery. Growth curves for these two microorganisms were similar, each approaching stationary growth at 95 h (10.7 g [dry weight] of cells per liter). Typically, the amounts of protein obtained by cell breakage ranged between 15 and 35 mg/ml.

Table 1 shows the specific activities of HK, PGM, UDPGP, and UDPGO in cell extracts of both *Streptomyces* cultures taken at 98 h during the chromomycin-producing, stage II culture. In this experiment, all enzymes were detected in both culture extracts. However, strain MRS202 contained twice the levels of HK and PGM specific activities for *S. griseus* ATCC 13273. Comparisons of these same enzyme activities in chromomycin A_3 -negative mutants showed that cultures incapable of producing the antibiotics lacked measurable UDPGO activ-

TABLE 1. Specific activities of HK, PGM, UDPGP, and UDPGO in 98-h *S. griseus* ATCC 13273, *Streptomyces* sp. strain MRS202, and non-antibiotic-producing mutant culture extracts^a

<i>Streptomyces</i> strain	Sp act (U/mg of protein)				
	HK	PGM	UDPGP	UDPGO (UDPG)	UDPGO (GDPG)
13273	0.024	0.055	0.024	0.010	0.007
MRS202	0.039	0.114	0.021	0.010	0.008
A_3^- mutants					
ASFz	0.098	0.247	0.055		
AMY	0.137	0.542	0.045		

^a Assays were conducted in duplicate, with a variation of no more than 3% for any sample.

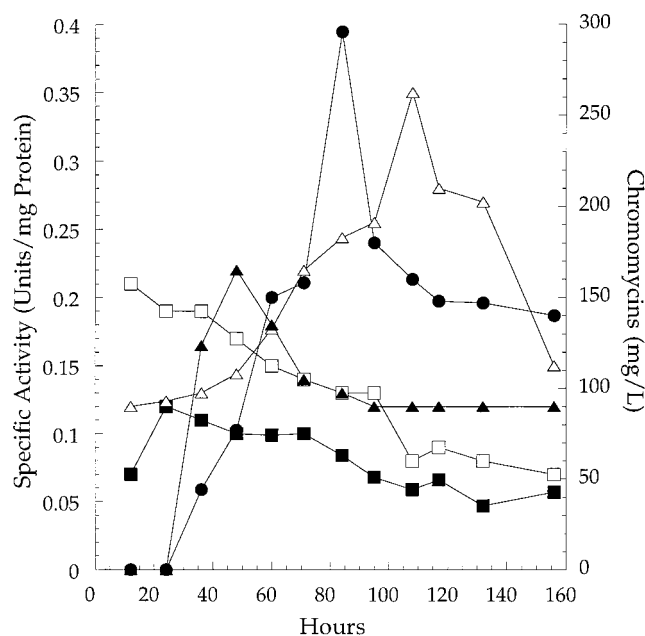


FIG. 3. Relationships of HK (■), PGM (△), UDPGP ($\times 10$) (□), and UDPGO ($\times 10$) (▲) to production of chromomycin A_3 (●) by *Streptomyces* sp. strain MRS202. The results are the averages of duplicate assays within a deviation of no more than 4%.

ity. Interestingly, the specific activities of UDPGP in two antibiotic-negative mutants (45 and 55 mU/mg of protein) were more than twice as high as those observed in antibiotic-producing cultures. These results indicate a positive correlation between UDPGO activity and antibiotic formation and a negative correlation between UDPGP activity and antibiotic formation.

One aim of this work was to determine possible relationships between these initial steps of glucose activation and antibiotic biosynthesis. Therefore, we compared the temporal relationships among HK, PGM, UDPGP, and UDPGO expressed activities and chromomycin biosynthesis for *Streptomyces* sp. strain MRS202. The results are shown in Fig. 3. Traces of chromomycins were evident in 24-h cultures, increased to 150 mg/liter at 60 h, and reached a peak concentration of 300 mg/liter at 84 h before decreasing to about 150 mg/liter thereafter. PGM specific activities were measured at 0.125 U/mg of protein at 12 h and gradually increased to a peak level of 0.35 U/mg of protein at 108 h before declining again. HK activity was highest at 24 h (0.13 U/mg of protein), declined to about 0.1 U/mg of protein by 48 h, and gradually decreased thereafter. Measured specific activities for UDPGP and UDPGO were much less than those for HK and PGM. UDPGP started at 0.02 U/mg of protein at 12 h, remained the same until 48 h, and then gradually declined to about half that level at 108 h and thereafter. UDPGO, however, was undetectable at 24 h, reached a peak of 0.025 U/mg of protein by 48 h, and then gradually declined to 0.017 U/mg of protein by 108 h, where it remained. Interestingly, UDPGO peak activity preceded antibiotic peak production by about 24 h, and the gradual decline in UDPGO activity likewise preceded the gradual decline in antibiotic levels by about 24 h. These results link expressed UDPGO activity to antibiotic production in this streptomycete. Furthermore, these results confirm the involvement of the enzymes indicated in Fig. 2 in glucose activation by *Streptomyces* sp. strain MRS202.

Since UDPGP apparently is a centrally important enzyme in glucose activation in *Streptomyces* sp. strain MRS202, several experiments were developed to confirm the presence of the reaction product, UDPG, and to rule out the involvement of other nucleotide pyrophosphorylases in the glucose activation process. UDPG levels in cell extracts were determined with UDPG dehydrogenase (24), which oxidizes UDPG to UDP glucuronate and concomitantly reduces NAD^+ . The preparations were also analyzed by HPLC (solvent system 1). Incubation mixtures all contained cell extract plus G-6-P, G-1-P, and one of the following triphosphonucleotides: UTP, GTP, dTTP, ATP, or CTP. Incubations containing inorganic pyrophosphatase, UDPGP, and PGM were evaluated as controls. UDPG was formed in cell extract preparations amended with UTP and either G-1-P or G-6-P, showing that endogenous PGM and G-1,6-diP were present in cell extracts. However, when ATP, dTTP, CTP, or GTP was added to cell extracts and incubated with G-1-P, no corresponding ADPG, dTDPG, CDPG, or GDPG was detected. These results indicated that either there were no corresponding ADPG, GDPG, CDPG, and dTDPG pyrophosphorylases or that *Streptomyces* UDPGP could not use ATP, dTTP, CTP, and GTP as substrates.

Using $100,000 \times g$ supernatants of MRS202 and *S. griseus* cell extracts, the optimum pH of UDPGO was determined to be pH 7.5. Crude UDPGO is stable below 45°C for 1 h without significant loss of activity, thus permitting analysis of its substrate range. The apparent K_m and V_{\max} values of UDPG for UDPGO were determined to be $50 \mu\text{M}$ and $23 \text{ nM min}^{-1} \text{ mg}^{-1}$, respectively, whereas the K_m (NAD^+) was $100 \mu\text{M}$. V_{\max} was lower than that for the *E. coli* enzyme ($7 \mu\text{M min}^{-1} \text{ mg}^{-1}$) (8). Comparison of the *Streptomyces* and *E. coli* V_{\max}/K_m values for UDPGO (4.6×10^{-4} and 1.66×10^{-3} , respectively) reveals that the *Streptomyces* sp. enzyme is 28 times less efficient than the *E. coli* enzyme and 33 times less efficient than TDPG oxidoreductase from *Saccharopolyspora erythraea* (28). Unlike UDPGO in other bacteria, the enzyme from *Streptomyces* sp. was active when CDPG, GDPG, or TDPG was substituted for UDPG. K'_m values for GDPG, CDPG, and TDPG were 58, 74, and $118 \mu\text{M}$, respectively, with the *Streptomyces* UDPGO. No activity could be detected with ADPG. The V'_{\max} values for CDPG, TDPG, and GDPG were 1.9, 2.7, and $3.2 \text{ nM min}^{-1} \text{ mg}^{-1}$, respectively. Comparison of V'_{\max} and K'_m of UDPG (determined as $50 \mu\text{M min}^{-1} \text{ mg}^{-1}$ and 23 nM , respectively) with those of GDPG (8.4 times slower), CDPG (18 times slower), and TDPG (20 times slower) indicates that UDPG is the preferred substrate.

In summary, this work has demonstrated that activities for HK, PGM, UDPGP, and UDPG-4,6-dehydratase (also known as UDPGO) occur in cell extracts of *Streptomyces* sp. strain MRS202 and *S. griseus* (ATCC 13273). G-6-P plays a central role in glycolysis, and it is a key intermediate in the oxidation and fermentation of glucose as an energy source. The nucleoside diphosphate derivative of glucose, UDPG, is an activated form of the sugar important in anabolic events, such as the polysaccharide and cell wall biosynthesis, and as a precursor for other nucleoside diphosphate sugars. Such sugar nucleotides are also implicated in the biosynthesis of secondary metabolites, such as the chromomycins. Both UDPGP and UDPGO were partially purified from MRS202 (results not shown here). Among the enzymes examined, it was observed that UDPGO activities appear to be correlated with the production of chromomycins. Thus, the pathway of sugar oxidation likely involves the formation of UDP-4-keto-6-deoxyglucose via G-6-P, G-1-P, and UDPG, catalyzed by HK, PGM, UDPGP, and UDPGO, respectively.

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