Triple Hydroxylation of Tetracenomycin A2 to Tetracenomycin C in Streptomyces glaucescens

OVEREXPRESSION OF THE tcmG GENE IN STREPTOMYCES LIVIDANS AND CHARACTERIZATION OF THE TETRACENOMYCIN A2 OXYGENASE*

Ben Shen† and C. Richard Hutchinson‡‡

From the †School of Pharmacy and the ‡Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Nucleotide sequence analysis of the tcmG gene has suggested that the TcmG protein is responsible for the triple-hydroxylation of tetracenomycin (Tcm) A2 to Tcm C in Streptomyces glaucescens (Decker, H., Motamedi, H., and Hutchinson, C. R. (1993) J. Bacterial. 175, 3876–3886). The heterologous expression of the tcmG gene in Streptomyces lividans and the purification and characterization of TcmG protein, which we have named Tcm A2 oxygenase, are described here. NH₂-terminal amino acid analysis of the purified enzyme led to the revision of the translational start site of tcmG to a TTG codon, 33 base pairs downstream of the GTG site assigned initially on the basis of nucleotide sequence analysis. Tcm A2 oxygenase is a monomeric protein in solution and contains 1 mol of non-covalently bound FAD; the apoenzyme can be partially reconstituted in vitro by addition of FAD. Tcm A2 oxygenase exhibits an optimal pH of 9.0–9.5 and prefers NADPH over NADH as an electron donor. The apparent Kₘ of the enzyme for Tcm A2, NADH, and NADPH are 1.81 ± 0.38, 260 ± 19, and 82.1 ± 17 μM, respectively, and the apparent Vₘₐₓ for the reaction is 14.7 ± 1.1 nmol Tcm C/min·mg. Purification and characterization of Tcm A2 oxygenase provide direct evidence to support the notion that the angular hydroxy groups of naphthacenequinones like Tcm C are introduced from ¹⁸O₂ via a mono- or dioxygenase process.

Tcm 1, a polyketide antitumor antibiotic, was first isolated from Streptomyces glaucescens in 1979 (1) and then reisolated from Streptomyces H-881 in 1984 (2). Its absolute stereochemistry was established in 1992 (3) as the 4R, 4aR, 12aR configuration (Fig. 1A). Together with elloramycin (4, 5), 2, tetracenomycin X (6), 3, dutomyacin (7, 4), and viridicatumtoxin (8, 5), I forms a small group of naphthacenequinones with unique structural feature of the highly hydroxylated semiquinone moiety (boxed in Fig. 1A).

We have been studying the biosynthesis of I as a model for the family of polyketides with fused aromatic rings. Previously, we established that 1 is formed from acetate and malonate and have elucidated all of its biosynthetic intermediates (Fig. 1B) (9–11). We also characterized several key enzymes of the pathway, including the Tcm F2 polyketide synthase (12–14), the Tcm F2 cyclase (15), and the Tcm F1 monooxygenase (16) and cloned (17) and analyzed (18–20) the nucleotide sequences of the complete gene cluster for the biosynthesis of 1 (Fig. 1B). These studies have provided detailed insights into the biochemistry and genetics of the biosynthesis of 1 in S. glaucescens, which can serve as a model for the formation of aromatic polyketides in general. During this work, we have suggested that the three cis-hydroxy groups at the 4, 4a, and 12a positions of 1 are introduced by hydroxylation of Tcm A2, 6, an unprecedented process catalyzed by the Tcm G protein (20). The latter idea was further supported by complementation experiments with the Tcm C non-producing mutant S. glaucescens WMH1089 that contains a 180 base pair deletion mutation that has been mapped to the tcmG gene (20); the production of 1 was restored upon transformation of the WMH1089 strain with pWHM126 that carries most of tcmG (20). Moreover, the results of in vitro ¹⁸O₂ feeding experiments have indicated that only the 4- and 12a-OH groups of 1 are derived from molecular oxygen, leaving the 4a-OH to arise presumably from water (6).

To extend our investigation of the biosynthesis of 1, in particular to understand the underlying enzymatic reaction mechanisms of the pathway, we studied the hydroxylation of 6 to 1 in vitro and report here the overexpression of tcmG in Streptomyces lividans and the purification and characterization of the Tcm A2 oxygenase. NH₂-terminal amino acid analysis of the purified enzyme has led to a revision of the tcmG translational start site to a TTG codon, 33 base pairs downstream of the GTG codon assigned initially on the basis of nucleotide sequence analysis (20). Our results establish the stoichiometry of the conversion of 6 to 1 and prove that this reaction is catalyzed by the enzyme encoded by tcmG. Tcm A2 oxygenase was found to be a monomeric flavoprotein containing 1 mol of non-covalently bound FAD and to require molecular oxygen and reduced nicotineamide cofactors.

EXPERIMENTAL PROCEDURES

General—UV-VIS spectra were recorded on a Hitachi U-3000 spectrophotometer (San Jose, CA). Refrigerated centrifugation was done in a Sorvall RC-5B superspeed centrifuge (Newtown, CT). A Pharmacia FPLC system was used for enzyme purification and all FPLC columns were purchased from Pharmacia Biotech Inc. HPLC was done with a Waters model 201 pump system (Marlborough, MA) and a Waters 484 variable wavelength absorbance detector. Enzyme incubations were performed in a GCA Precision shaking water bath (± 0.1 °C) (Precision Scientific Inc., Chicago, IL). Fermentations were carried out in a rotary shaker-incubator (Series 25, New Brunswick Scientific Co. Inc., Edison, NJ). Analytical TLC was done on precoated Keiselgel 60 SiO₂ glass plates (0.25 mm) and was visualized by long- and/or short-wave UV light.

Bacterial Strains, Plasmids, and Other Materials—The S. glaucescens type strains (9), S. lividans 1326 (24), and plasmid pWHM3 (25)

* This work was supported by National Institutes of Health Grant CA35381. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
† To whom correspondence should be addressed: School of Pharmacy, 225 N. Charter St., Madison WI 53706. Tel: 608-262-7582; Fax: 608-262-3134; E-mail: CRHUTCH@PCASTAFF.WISC.EDU.
‡‡ The abbreviations used are: Tcm, tetracenomycin; BSA, bovine serum albumin; DTT, dithiothreitol; FPLC, fast protein liquid chromatography; HPLC, high performance liquid chromatography; RBS, ribosome-binding site; PAGE, polyacrylamide gel electrophoresis; TLC, thin layer chromatography; kb, kilobases; PFR, polymerase chain reaction,
acetyl CoA
+ 9 malonyl CoA

TcmJ

TcmKLMN

TcmI

TcmH

TcmNOP

TcmG

Fig. 1. Tcm C and related naphthacenequinones (A) and biosynthetic pathway of Tcm C in S. glaucescens (B).

are described elsewhere; pSP72 and pGem7zf were obtained from Promega Corporation (Madison, WI). The ermE' promoter containing plasmids pWHM63, pWHM64, and pWHM65 were gifts from G. Meurer.1 Thiostrepton was obtained from S. lucidorum at the Squibb Institute for Medical Research (Princeton, NJ). Unless specified, common chemicals, restriction enzymes, DNA ligase, and other materials for recombinant DNA procedures were purchased from standard commercial sources and used as provided.

DNA Isolation and Manipulation—Plasmid DNA from Escherichia coli was prepared according to Lee and Rasheed (26). For plasmid DNA isolations from Streptomyces spp., the cells were lysed according to Hopwood et al. (27), then treated as described by Lee and Rasheed (26). Agarose gel electrophoresis, restriction enzyme digestion, DNA ligation, and preparation of competent E. coli DH5α cells and their transformation were performed by established methods (28). DNA was purified from agarose gels with the QIAEX kit as directed by the manufacturer (QIAGEN Inc., Chatsworth, CA). Protoplasts of Streptomyces spp. were prepared and transformed by the methods of Hopwood et al. (27).

Construction of pWHM68, pWHM72, and pWHM73—To prepare pWHM68, a 2.1-kb EcoRI-HindIII fragment from pWHM1018 (20) that contains tcmG was cloned into the same sites of pSP72 to give pWHM66. The latter was digested with HindIII and EcoRV and the later was digested with HindIII and EcoRV and the resulting 2.1-kb fragment was cloned into the HindIII-SmaI sites of pWHM63 to give pWHM65, from which a NsiI-XbaI fragment was moved into the XbaI-PstI sites of pWHM3 to yield pWHM68 (Table I).

To construct pWHM72 and pWHM73, polymerase chain reactions (PCR) were used with site-specifically modified oligodeoxynucleotides to generate a fragment of the NH₂ terminus of tcmG with a unique SphI site at an ATG translational start site. PCR was carried out using a Perkin Elmer model 480 thermal cycler (Oak Brook, IL) with Taq polymerase and buffer supplied by Promega Corporation. Oligodeoxynucleotide primers were synthesized on a model 391 DNA synthesizer (Applied Biosystems, Foster City, CA), purified on 8 μl urea, 16% polyacrylamide gels, and electroeluted from the gel slices. The PCR mixture consisted of 50 μl of the 2 x buffer (40 μM Tris-HCl, pH 8.3, 2.4 mM MgCl₂, 40 mM KCl, 0.2% Triton X-100), 2 μl of DNA (20 ng), 1 μl of each primer (5'-ACACCAAGCTTCTAGAGCATGCCCGTTTCCGACCGACCGAAA-3', 1.0 ng), 5 μl of formamide, 1 μl of BSA (1 mg/ml), 36 μl of H₂O. The reaction mixture was covered with three drops of mineral oil, boiled for 5 min, and placed at 70 °C. To this mixture was added 4 μl of the dNTP mixture (final concentrations for dATP and dTTP, 40 μM, respectively) and 1 μl of Taq polymerase (4.5 units). The PCR temperature program was as follows: 24 cycles of 40 s at 96.5 °C and then 2.5 min at 71 °C; after the last amplification cycle, 40 s at 97 °C and then 7 min at 71 °C. After cycle 12, an additional 0.5 μl of Taq polymerase (2.25 units) was added. The amplified 306-base pair fragment was purified by agarose gel electrophoresis, digested with HindIII and SphI, and ligated with the 4.2-kb HindIII-SphI fragment obtained from partial digestion of pWHM1018 to yield pWHM69. The tcmG gene was transferred from pWHM69 as a SphI-XbaI fragment into similar sites of pWHM64 and pWHM65 to give pWHM70 and pWHM71, from which the 2.1-kb EcoRI fragments were cloned into the same site of pWHM3 to yield pWHM72 and pWHM73, respectively (Table I).

Protein Analysis—Protein concentrations were determined by the Bradford (29) method with BSA as the calibration standard. Pure Tcm A2 oxygenase also was quantified by UV absorption at 280 nm where the molar absorbance index (ε₂₈₀ nm) is 75.7 M⁻¹ cm⁻¹ (this value was calculated from the amino acid sequence deduced for the apoenzyme from tcmG). The molecular weight of the enzyme subunit was determined by SDS-PAGE using the Life Technologies, Inc. protein molecular weight standards of myosin H-chain 200,000, phosphorylase b 97,400, BSA 66,000, ovalbumin 43,000, carbonic anhydrase 29,000, β-lactoglobulin 18,400, and lysozyme 14,500. SDS-PAGE was performed according to the method of Laemmli (30) or on the PhastSystem (Pharmacia) as described by the manufacturer and the gels were Coomassie Blue-stained (31). The abundance of each band was then quantified on a Molecular Dynamics model 300A Computing Densitometer (Sunnyvale, CA). The molecular weight of the native Tcm G was determined by gel filtration chromatography on a Superose 6 HR 10/30 column in 20 mM sodium phosphate, pH 7.2, 1 mM DTT, 150 mM NaCl with a flow rate of 0.4 ml/min and the column was calibrated with blue dextrin 2 × 10⁵, alcohol dehydrogenase, 150,000, BSA 66,000, carbonic anhydrase 29,000, and cytchrome c 12,400 purchased from Sigma.

Enzyme Assays—The substrate and authentic product 1 were isolated from S. glaucescens WHM1089 and S. glaucescens GLA0, respectively, and characterized as described elsewhere (1, 9, 10).
Typically, 250 μl of assay solution with 10% (v/v) dimethyl sulfoxide, consisting of 100 μM 6, 250 μM NADPH, and 1 μM DT in 50 mM ethanalamine-HCl buffer, pH 9.5, in the presence of enzyme (10–50 μl), was incubated at 25 °C. The assay was initiated by addition of 6 and terminated by addition of solid NaH₂PO₄ to saturation and extraction with EtOAc (2 × 400 μl). The EtOAc extracts were collected and concentrated in vacuo to dryness, then the residue was dissolved in 50–120 μl of methanol and analyzed by TLC or HPLC. SiO₂ plates were used and developed in CHCl₃/MeOH (95:5, v/v), under these conditions 6 and 1 have an Rf of 0.77 and 0.28 and, under UV light, display a characteristic yellow and blue fluorescence, respectively. This TLC method was used throughout the purification to monitor the enzyme activity qualitatively. Alternatively, a HPLC method was developed that provided a quantitative analysis of the enzymatic synthesis of 6 from 1. Assay samples were analyzed by HPLC on a Nova-Pak C₁₈ column (Waters) developed with a linear gradient from CH₃CN:H₂O/ AcOH (20:80:0.1%, v/v) to CH₃CN in 10 min followed by additional 5 min at 100% CH₃CN at a flow rate of 2 ml/min with UV detection at 280 nm. The column was calibrated with authentic 1 and 6 that, under these conditions, have retention times of 6.5 and 12.0 min, respectively.

The HPLC assay method was used in all studies with the following modifications. For the pH dependence study, the assays were performed in 50 mM Tris-HCl buffer, pH 6.5–9.0, and 50 mM ethanalamine-HCl buffer, pH 8.0–10.5, respectively, in the presence of 12 μg of TcmG. For determination of the kinetic parameters, the assays were done with a concentration of 6 varied from 0.5 to 30 μM, 1.0 mM NADPH, and 2.93 μg of TcmG, respectively, in 250 ml of assay solution with 10% (v/v) dimethyl sulfoxide, pH 7.2, 1 μM DT, 150 μM NaN₃, and applied to a Sephacryl S-200 HR column. The column was washed with a flow rate of 2 ml/min with a linear 60-ml gradient from 0 to 6.0 mM NaCl in the same buffer, and 2-ml fractions were collected.

TABLE I

| Plasmid    | Description                           | Refs. |
|------------|---------------------------------------|-------|
| pWHM1018   | tcmG gene behind the tcmG promoter in pUC19 | 20    |
| pWHM1019   | tcmG gene behind the tcmG promoter in pWHM3 | 20    |
| pWHM63     | ermE* promoter in pGem7zf              |       |
| pWHM64     | ermE* promoter engineered with the RBS of CCCAGGAGGT |       |
| pWHM65     | ermE* promoter engineered with the RBS of GAAAGGAGGT |       |
| pWHM66     | found in the melC gene from S. antibioticus in pGem7zf |       |
| pWHM66     | tcmG gene behind the tcmG promoter in pSP72 |       |
| pWHM67     | tcmG gene behind the tandem ermE* and tcmG promoters in pGem7zf |       |
| pWHM68     | tcmG gene behind the tandem ermE* and tcmG promoters in pWHM3 |       |
| pWHM69     | tcmG gene behind the ermE* promoter in pUC19 |       |
| pWHM70     | tcmG gene behind the ermE* promoter with the RBS of CCCAGGAGGT in pUC19 |       |
| pWHM71     | tcmG gene behind the ermE* promoter with the RBS of GAAAGGAGGT in pUC19 |       |
| pWHM72     | tcmG gene behind the ermE* promoter with the RBS of CCCAGGAGGT in pWHM3 |       |
| pWHM73     | tcmG gene behind the ermE* promoter with the RBS of GAAAGGAGGT in pWHM3 |       |

Typically, 250 μl of assay solution with 10% (v/v) dimethyl sulfoxide, consisting of 100 μM 6, 250 μM NADPH, and 1 μM DT in 50 mM ethanalamine-HCl buffer, pH 9.5, in the presence of enzyme (10–50 μl), was incubated at 25 °C. The assay was initiated by addition of 6 and terminated by addition of solid NaH₂PO₄ to saturation and extraction with EtOAc (2 × 400 μl). The EtOAc extracts were collected and concentrated in vacuo to dryness, then the residue was dissolved in 50–120 μl of methanol and analyzed by TLC or HPLC. SiO₂ plates were used and developed in CHCl₃/MeOH (95:5, v/v), under these conditions 6 and 1 have an Rf of 0.77 and 0.28 and, under UV light, display a characteristic yellow and blue fluorescence, respectively. This TLC method was used throughout the purification to monitor the enzyme activity qualitatively. Alternatively, a HPLC method was developed that provided a quantitative analysis of the enzymatic synthesis of 6 from 1. Assay samples were analyzed by HPLC on a Nova-Pak C₁₈ column (Waters) developed with a linear gradient from CH₃CN:H₂O/ AcOH (20:80:0.1%, v/v) to CH₃CN in 10 min followed by additional 5 min at 100% CH₃CN at a flow rate of 2 ml/min with UV detection at 280 nm. The column was calibrated with authentic 1 and 6 that, under these conditions, have retention times of 6.5 and 12.0 min, respectively.

The HPLC assay method was used in all studies with the following modifications. For the pH dependence study, the assays were performed in 50 mM Tris-HCl buffer, pH 6.5–9.0, and 50 mM ethanalamine-HCl buffer, pH 8.0–10.5, respectively, in the presence of 12 μg of TcmG. For determination of the kinetic parameters, the assays were done with a concentration of 6 varied from 0.5 to 30 μM, 1.0 mM NADPH, and 2.93 μg of TcmG, respectively, in 250 ml of assay solution with 10% (v/v) dimethyl sulfoxide, pH 7.2, 1 μM DT, 150 μM NaN₃, and applied to a Sephacryl S-200 HR column. The column was washed with a flow rate of 2 ml/min with a linear 60-ml gradient from 0 to 6.0 mM NaCl in the same buffer, and 2-ml fractions were collected.

Preparation of Aromatic Alcohol from Its Aromatic Alcohol Dehydrogenase—To prepare apo-TcmG, 1 ml of pure TcmG protein (1.46 mg) was dialyzed against 200 ml of 100 mM potassium phosphate buffer, pH 4.0, 2.0 mM KBr for 2 days with five buffer changes (35, 36). This sample was then dialyzed against 200 ml of 25 mM Tris-HCl, pH 8.0, 1 mM DTW for 2 days with five buffer changes to yield the apoenzyme that was used directly for the NH₂-terminal amino acid sequence determination according to their molar absorbance indexes of ε₂₈₀ = 11.3 mM⁻¹ cm⁻¹ and ε₆₅₀ = 12.2 mM⁻¹ cm⁻¹, respectively.

Preparation of Apo-Tcm A2 Oxygenase and Its In Vitro Reconstitution—To prepare apo-TcmG, 1 ml of pure TcmG protein (1.46 mg) was dialyzed against 200 ml of 100 mM potassium phosphate buffer, pH 4.0, 2.0 mM KBr for 2 days with four buffer changes (35, 36). This sample was then dialyzed against 200 ml of 25 mM Tris-HCl, pH 8.0, 1 mM DTW for 2 days with five buffer changes to yield the apoenzyme that was used directly for the NH₂-terminal amino acid sequence determination according to their molar absorbance indexes of ε₂₈₀ = 11.3 mM⁻¹ cm⁻¹ and ε₆₅₀ = 12.2 mM⁻¹ cm⁻¹, respectively.

Preparation of Apo-Tcm A2 Oxygenase and Its In Vitro Reconstitution—To prepare apo-TcmG, 1 ml of pure TcmG protein (1.46 mg) was dialyzed against 200 ml of 100 mM potassium phosphate buffer, pH 4.0, 2.0 mM KBr for 2 days with four buffer changes (35, 36). This sample was then dialyzed against 200 ml of 25 mM Tris-HCl, pH 8.0, 1 mM DTW for 2 days with five buffer changes to yield the apoenzyme that was used directly for the NH₂-terminal amino acid sequence determination according to their molar absorbance indexes of ε₂₈₀ = 11.3 mM⁻¹ cm⁻¹ and ε₆₅₀ = 12.2 mM⁻¹ cm⁻¹, respectively.

Preparation of Apo-Tcm A2 Oxygenase and Its In Vitro Reconstitution—To prepare apo-TcmG, 1 ml of pure TcmG protein (1.46 mg) was dialyzed against 200 ml of 100 mM potassium phosphate buffer, pH 4.0, 2.0 mM KBr for 2 days with four buffer changes (35, 36). This sample was then dialyzed against 200 ml of 25 mM Tris-HCl, pH 8.0, 1 mM DTW for 2 days with five buffer changes to yield the apoenzyme that was used directly for the NH₂-terminal amino acid sequence determination according to their molar absorbance indexes of ε₂₈₀ = 11.3 mM⁻¹ cm⁻¹ and ε₆₅₀ = 12.2 mM⁻¹ cm⁻¹, respectively.

Preparation of Apo-Tcm A2 Oxygenase and Its In Vitro Reconstitution—To prepare apo-TcmG, 1 ml of pure TcmG protein (1.46 mg) was dialyzed against 200 ml of 100 mM potassium phosphate buffer, pH 4.0, 2.0 mM KBr for 2 days with four buffer changes (35, 36). This sample was then dialyzed against 200 ml of 25 mM Tris-HCl, pH 8.0, 1 mM DTW for 2 days with five buffer changes to yield the apoenzyme that was used directly for the NH₂-terminal amino acid sequence determination according to their molar absorbance indexes of ε₂₈₀ = 11.3 mM⁻¹ cm⁻¹ and ε₆₅₀ = 12.2 mM⁻¹ cm⁻¹, respectively.
assayed to determine the residual enzyme activity. For in vitro reconstitution of the apoenzyme, a 500-µl solution containing 1.65 nmol of the apoenzyme, 16.5 nmol of either FAD or FMN, respectively, in 25 mM Tris-HCl, pH 8.0, 1 mM DTT was incubated on ice for 1.5 h (35, 36); 1.65 nmol of the holoenzyme was similarly treated with FAD or FMN to ensure no contamination of the apoenzyme. A distinct band migrating with a size of 60,000 Da was observed in all extracts from either the reconstituted enzyme was assayed directly by the HPLC method with the thiol acceptor (25) so as to place the expression of TcmG under the conditions described above to ensure no contamination of TcmG oxygenase activity among several common flavin derivatives in the commercial materials (34). The resulting reconstituted enzyme was assayed directly by the HPLC method without attempting to remove the excess FAD or FMN.

RESULTS

Heterologous Expression of the tcmG Gene in S. lividans—Since Tcm A2 oxygenase activity was not detected in cellular extracts from either the S. glaucescens GLA.0 wild-type strain or the WMH1094 (20) Tcm C non-producing mutant that bio-transformed 6 to 1 effectively in vivo, we expressed the tcmG gene in S. lividans to facilitate the isolation and characterization of Tcm A2 oxygenase. pWHM1019, pWHM72, and pWHM73 were made in the high copy number vector pWHM3 (25) so as to place the expression of TcmG under the control of the tcmG promoter (21) with the tcmG RBS from pWHM3 (20) or of the ermE* promoter (12) in combination with a RBS from either Streptomycetes antibioticus (37) or S. lividans (38), since it is known that the ermE* promoter displays the strongest activity among several common Streptomycetes promoters studied (39) and that the sequence of the RBS can also have a distinct effect on the level of gene expression. pWHM68 was constructed to examine the effect of placing the ermE* and tcmG promoters in tandem on the level of expression of tcmG. All four plasmids were introduced by transformation into S. lividans 1326 (24), and the levels of tcmG expression were assayed by SDS-PAGE of cell-free extracts prepared and analyzed as described under "Experimental Procedures." A distinctive band migrating with a size of 60,000 Da was observed in all four cases (Fig. 2A, lanes 1–4); this band was absent in a sample from the control culture of S. lividans (pWHM3) (Fig. 2A, lane 5). The apparent size of the expressed protein is consistent with the size of 61,694 Da predicted from the nucleotide sequence of tcmG (20). Whereas the abundance of TcmG was approximately the same from the plasmids in which tcmG expression was under the control of either ermE* or tcmG promoter alone (Fig. 2A, abundance in lanes 2–4 = 1, 0.89, and 0.89), the tandem ermE*:tcmG promoter system resulted in approximately 5-fold higher level of TcmG than any single promoter counterpart (Fig. 2A, abundance in lanes 1–4 = 4.8, 1.0, 0.89, 0.89).

Purification of the TcmG Protein from S. lividans (pWHM68)—Since S. lividans (pWHM68) expressed tcmG most effectively among the constructs tested, a cell-free extract was prepared from this recombinant strain for the purification of TcmG. The enzyme activity was first treated with FAD or FMN to place the expression of TcmG

\[ \text{TcmG} \]

\[ \text{KDa} \]

\[ \begin{array}{c|cccc}
\text{Step} & \text{Protein} & \text{Activity} & \text{Specific} & \text{Yield} & \text{Purification} \\
& \text{mg} & \text{nmol/} & \text{nmol/} & \% & x-
\end{array} \]

\[ \begin{array}{cccccc}
\text{Cell-free extract} & 865 & 188 & 0.217 & 100 & 1.00 \\
\text{NADPH} & 570 & 162 & 0.284 & 86.2 & 1.31 \\
\text{Mono Q HR 10/10} & 81.9 & 140 & 1.71 & 74.5 & 8.16 \\
\text{Pheny} & 32.2 & 138 & 4.29 & 73.4 & 19.8
\end{array} \]

approximately 5-fold higher level of TcmG than any single promoter counterpart (Fig. 2A, abundance in lanes 1–4 = 4.8, 1.0, 0.89, 0.89).

Purification of the TcmG Protein from S. lividans (pWHM68)—Since S. lividans (pWHM68) expressed tcmG most effectively among the constructs tested, a cell-free extract was prepared from this recombinant strain for the purification of TcmG. The enzyme activity was first treated with FAD or FMN to place the expression of TcmG

\[ \text{TcmG} \]

\[ \text{KDa} \]

\[ \begin{array}{c|cccc}
\text{Step} & \text{Protein} & \text{Activity} & \text{Specific} & \text{Yield} & \text{Purification} \\
& \text{mg} & \text{nmol/} & \text{nmol/} & \% & x-
\end{array} \]

\[ \begin{array}{cccccc}
\text{Cell-free extract} & 865 & 188 & 0.217 & 100 & 1.00 \\
\text{NADPH} & 570 & 162 & 0.284 & 86.2 & 1.31 \\
\text{Mono Q HR 10/10} & 81.9 & 140 & 1.71 & 74.5 & 8.16 \\
\text{Pheny} & 32.2 & 138 & 4.29 & 73.4 & 19.8
\end{array} \]

approximately 5-fold higher level of TcmG than any single promoter counterpart (Fig. 2A, abundance in lanes 1–4 = 4.8, 1.0, 0.89, 0.89).

RESULTS

Heterologous Expression of the tcmG Gene in S. lividans—Since Tcm A2 oxygenase activity was not detected in cellular extracts from either the S. glaucescens GLA.0 wild-type strain or the WMH1094 (20) Tcm C non-producing mutant that bio-transformed 6 to 1 effectively in vivo, we expressed the tcmG gene in S. lividans to facilitate the isolation and characterization of Tcm A2 oxygenase. pWHM1019, pWHM72, and pWHM73 were made in the high copy number vector pWHM3 (25) so as to place the expression of TcmG under the control of the tcmG promoter (21) with the tcmG RBS from pWHM3 (20) or of the ermE* promoter (12) in combination with a RBS from either Streptomycetes antibioticus (37) or S. lividans (38), since it is known that the ermE* promoter displays the strongest activity among several common Streptomycetes promoters studied (39) and that the sequence of the RBS can also have a distinct effect on the level of gene expression. pWHM68 was constructed to examine the effect of placing the ermE* and tcmG promoters in tandem on the level of expression of tcmG. All four plasmids were introduced by transformation into S. lividans 1326 (24), and the levels of tcmG expression were assayed by SDS-PAGE of cell-free extracts prepared and analyzed as described under "Experimental Procedures." A distinctive band migrating with a size of 60,000 Da was observed in all four cases (Fig. 2A, lanes 1–4); this band was absent in a sample from the control culture of S. lividans (pWHM3) (Fig. 2A, lane 5). The apparent size of the expressed protein is consistent with the size of 61,694 Da predicted from the nucleotide sequence of tcmG (20). Whereas the abundance of TcmG was approximately the same from the plasmids in which tcmG expression was under the control of either ermE* or tcmG promoter alone (Fig. 2A, abundance in lanes 2–4 = 1, 0.89, and 0.89), the tandem ermE*:tcmG promoter system resulted in approximately 5-fold higher level of TcmG than any single promoter counterpart (Fig. 2A, abundance in lanes 1–4 = 4.8, 1.0, 0.89, 0.89).

Purification of the TcmG Protein from S. lividans (pWHM68)—Since S. lividans (pWHM68) expressed tcmG most effectively among the constructs tested, a cell-free extract was prepared from this recombinant strain for the purification of TcmG. The enzyme activity was first treated with FAD or FMN to place the expression of TcmG

\[ \text{TcmG} \]

\[ \text{KDa} \]

\[ \begin{array}{c|cccc}
\text{Step} & \text{Protein} & \text{Activity} & \text{Specific} & \text{Yield} & \text{Purification} \\
& \text{mg} & \text{nmol/} & \text{nmol/} & \% & x-
\end{array} \]

\[ \begin{array}{cccccc}
\text{Cell-free extract} & 865 & 188 & 0.217 & 100 & 1.00 \\
\text{NADPH} & 570 & 162 & 0.284 & 86.2 & 1.31 \\
\text{Mono Q HR 10/10} & 81.9 & 140 & 1.71 & 74.5 & 8.16 \\
\text{Pheny} & 32.2 & 138 & 4.29 & 73.4 & 19.8
\end{array} \]
A. Originally assigned TcmG>DMeta-P-V-S-D-R-P-K-G-C-I-

5'-ATCGCAGGGAGTACTCCTGTCGCCGGTTTCCGACCAGCCGGGAAAGCTCATTTTCTCACCAGAAGTAAGTCTCGGCT-3'

Revised TcmG>DMeta-S-T-E-E-V-P-V-L-I-V-

B. 1 STEEPVFLVQGGLKLSSAALEFLQHIVSCRLVESQIOTVRTRSGIQFMELBLSGQVLYEETFIRLQEVQAMRELOQPAQIQMVVRIRAKIEDL

101 LEHUVTRRSPFVQCPQDRRLPSILDDDVRQGAFLIDTTSQVITATQDQGQKVTRVRYLIADQDRVSPVRQITAT

201 GHEEGDAMSRLCPKLDLRTVQKRQPVLCQISGDQVQLQEPQDVQLQLFQEDQKSFQFQPQFSPSFQEQIQQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQIQI

conditions studied. As summarized in Table III, the enzyme utilizes either NADH or NADPH as electron donors and requires molecular oxygen; removing O₂ by exchange with nitrogen inhibited the hydroxylation completely, as did heat denaturation.

**pH Dependence**—Tcm A2 oxygenase displayed an optimal pH of 9.0−9.5 in 50 mM Tris-HCl or 50 mM ethanolamine-HCl buffer, as shown in Fig. 4. While a decrease of 1 unit below the optimal pH caused an approximately 50% loss of the specific activity, an increase of 1 unit above the optimal pH resulted in complete loss of the enzyme activity. It is not known, however, if the loss of enzyme activity at pH > 9.5 resulted from deprotonation of specific amino acid residues at the active site, from denaturation of the protein, or from decomposition of NADPH.

**Prosthetic Group Investigation**—Most of the known oxygenases have either a flavin or heme as their prosthetic group or require a metal ion for the activation of molecular oxygen. Since analysis of the nucleotide sequence of tcmG has revealed a conserved domain for flavin binding (20) and a flavin has characteristic absorption maxima at 375 and 450 nm (36), we determined the UV-VIS absorption spectrum of the purified Tcm A2 oxygenase (Fig. 5). These data show that Tcm A2 oxygenase is a flavoenzyme. To further identify the nature of this flavin prosthetic group, a solution of Tcm A2 oxygenase (224 μg) was heat denatured to release the non-covalently bound flavin (33), which then was analyzed by HPLC on a C₁₈ column. Under the given conditions, most of the known flavin derivatives such as FAD and FMN are well separated (34), as shown in Fig. 6A. Fig. 6B shows that the prosthetic group released from Tcm A2 oxygenase is FAD, which was further confirmed by co-chromatography with a mixture of authentic FAD and FMN (Fig. 6C). From the results of HPLC analysis, calibrated with authentic FAD, it was established that the molar ratio of apo-TcmG:FAD is 1:1. This value agrees reasonably well with a 1:0.73 ratio of apo-TcmG:FAD, determined spectroscopically based on the molar absorbance indexes of ε⁺²⁵₅ and ε⁺₃₆₅, respectively.

**Preparation of Apo-Tcm A2 Oxygenase and Its in Vitro Reconstitution with FAD**—After establishing that Tcm A2 oxygenase contains 1 mol of non-covalently bound FAD as a prosthetic group, we explored ways to prepare the apo-Tcm A2 oxygenase. The best results were obtained by dialysis of Tcm A2 oxygenase in 100 mM potassium phosphate buffer, pH 4.0, in the presence of 2 mM KCl (35, 36). The protein was completely denatured by this treatment as indicated by its precipitation and was resolubilized and presumably refolded by subsequent dialysis in 25 mM Tris-HCl, pH 8.0, 1 mM DTT. The apoenzyme was colorless, in contrast to the characteristic yellow color of the holoenzyme, suggesting the removal of the flavin prosthetic group, and possessed very little of the initial enzyme activity (Table IV). The apo-Tcm A2 oxygenase could be partially reconstituted in vitro (35, 36), but only by FAD; addition of either FAD or FMN to the holo-Tcm A2 oxygenase under parallel conditions resulted in a very small change in activity.
**Tetracenomycin A2 oxygenase from S. glaucescens**

In *vitro* reconstitution of apo-Tcm A2 oxygenase with FAD and FMN

| Assay description | Relative activity* |
|-------------------|-------------------|
| Holoenzyme        | 100               |
| Holoenzyme + FAD  | 115               |
| Holoenzyme + FMN  | 87                |
| Apoenzyme         | <4                |
| Apoenzyme + FAD   | 23                |
| Apoenzyme + FMN   | <4                |

* The complete assay solution of 250 μl with 10% (v/v) dimethyl sulfoxide consisted of 100 mM 6, 1.0 mM NADPH, and 1 mM DTT in 50 mM ethanolamine-Cl buffer, pH 8.0, in the presence of 5.85 μg of holo- or apo-Tcm A2 oxygenase with/without FAD or FMN, respectively, was incubated at 25 °C for 10 min and then analyzed by the HPLC method described under “Experimental Procedures.”

The purification and characterization of Tcm A2 oxygenase supports the hypothesis that the triple hydroxylation of 6 to 1 is catalyzed by a single enzyme. This enzyme requires molecular oxygen and is able to use either NADH or NADPH. Since the apparent $V_{\text{max}}/K_m$ for NADPH (0.179) is more than 3-fold larger than that for NADH (0.0565), we conclude that Tcm A2 oxygenase prefers NADPH under physiological conditions.

The exact mechanism of the Tcm A2 oxygenase catalyzed hydroxylation of 6 to 1 is not clear yet since the data reported here do not discriminate between a monoxygenase and a dioxygenase mechanism. As proposed in Fig. 7 (route a), two of the three oxygens could be introduced stepwise from molecular oxygen if the enzyme acts like a monoxygenase. The first monoxygenase activity could hydroxylate 8 to hydroquinone 7 that could be further oxidized by the second monoxygenase activity to yield epoxquinone 8; cis opening of oxirane ring by a H$_2$O molecule could introduce the third oxygen to yield dihydroxquinone 9 that could be finally reduced to 1. In contrast, two of the three oxygens could also be introduced in a concerted fashion from molecular oxygen if the enzyme acts as a dioxygenase (Fig. 7, route b) where a likely stable intermediate would be the epoxysemiquinone 10; cis opening of its oxirane ring by a H$_2$O molecule could introduce the third oxygen to yield 1. Both mechanisms are consistent with the results of an in *vivo* $^{18}$O$_2$ feeding experiment that has demonstrated that the oxygens of the 4-OH and 12a-OH groups come from molecular oxygen and the 4a-OH group presumably comes from H$_2$O (6).

**DISCUSSION**

The enzymatic mechanism for the introduction of angular hydroxy groups like 4a-OH and 12a-OH of 1 into many other naphthacenequinone, angucycline, and anthracycline antibiotics (4–8, 42) is unknown, and the origins of such groups have been studied previously only by in *vivo* feeding experiments with $^{18}$O$_2$ or $^{18}$O-containing precursors (6, 8, 43–45) or by inhibition of the oxygenase with P-450 inhibitors (46, 47). At least three pathways can be proposed for their introduction. They could simply be retained from the carbonyl groups of polyketide precursors, such as acetate, malonate, etc., without going through an aromatic intermediate like 6, as in the urdamycins (42) or 5, whose 4a-OH was found to be derived from acetate (8). Alternatively, they could be introduced late in the biosynthetic pathway by an oxygenase, as proposed for 1 in Fig. 7, acting as either a monoxygenase (route a) or a dioxygenase (route b).

**TABLE IV**

| Assay description | Relative activity* |
|-------------------|-------------------|
| Holoenzyme        | 100               |
| Holoenzyme + FAD  | 115               |
| Holoenzyme + FMN  | 87                |
| Apoenzyme         | <4                |
| Apoenzyme + FAD   | 23                |
| Apoenzyme + FMN   | <4                |

FIG. 5. UV-VIS absorbance spectrum of the Tcm A2 oxygenase (1.12 mg/ml in 25 mM Tris-Cl, pH 8.0).

FIG. 6. HPLC analysis of FAD prosthetic group dissociated from the Tcm A2 oxygenase. A, a mixture of authentic FAD and FMN; B, the flavin prosthetic group dissociated from the Tcm A2 oxygenase; C, the dissociated flavin prosthetic group was co-chromatographed with the mixture of authentic FAD and FMN analyzed in A.

Kinetics—Assuming that the O$_2$ concentration was constant in the assay solution, kinetic analyses were carried out on the basis of a pseudo-first-order treatment with a steady-state approach. Thus, the effect of the initial concentration of 6 on the formation of 1 was determined at the concentration of NADPH $\geq 10 K_m^\text{apo-Tcm}$. The effect of NADH or NADPH was determined at the concentration of 6 $\geq 10 K_m^\text{apo-Tcm}$. Velocities were then fitted to the Michaelis-Menten equation (32) and the apparent $V_{\text{max}}$ for 6, NADH, and NADPH were found to be 1.81 ± 0.38, 260 ± 19, and 82.1 ± 17 nmol, respectively, with an apparent $V_{\text{max}}$ of 14.7 ± 1.1 nmol Tcm C/min-mg.

The exact mechanism of the Tcm A2 oxygenase catalyzed hydroxylation of 6 to 1 is not clear yet since the data reported here do not discriminate between a monoxygenase and a dioxygenase mechanism. As proposed in Fig. 7 (route a), two of the three oxygens could be introduced stepwise from molecular oxygen if the enzyme acts like a monoxygenase. The first monoxygenase activity could hydroxylate 8 to hydroquinone 7 that could be further oxidized by the second monoxygenase activity to yield epoxquinone 8; cis opening of oxirane ring by a H$_2$O molecule could introduce the third oxygen to yield dihydroxquinone 9 that could be finally reduced to 1. In contrast, two of the three oxygens could also be introduced in a concerted fashion from molecular oxygen if the enzyme acts as a dioxygenase (Fig. 7, route b) where a likely stable intermediate would be the epoxysemiquinone 10; cis opening of its oxirane ring by a H$_2$O molecule could introduce the third oxygen to yield 1. Both mechanisms are consistent with the results of an in *vivo* $^{18}$O$_2$ feeding experiment that has demonstrated that the oxygens of the 4-OH and 12a-OH groups come from molecular oxygen and the 4a-OH group presumably comes from H$_2$O (6). The monoxygenase pathway (Fig. 7, route a) is supported by the amino acid sequence similarity between TcmG (20) and other bacterial hydroxylases, such as those found in the oxytetracycline producer *Streptomyces rimosus* (49) and the
derivatives are utilized as cofactors in stable than the holoenzyme, yet the holoenzyme can be reconstructed by FAD only, albeit to a low degree (Table olivaceus 2.) The latter observation re-enforces the conclusion that Tcm identified (36). It is known, however, that unusual flavin de-FMN are the most common forms of the flavin prosthetic group of oxygenase from preparation of apo-Tcm protein upon heat denaturation (33). These facts led to the mechanism similar to route b has recently been established for the latter enzyme does not possess any prosthetic group or require the vitamin K-dependent carboxylase (53, 541, although the NAD(P)H cofactors.

Many bacterial oxygenases are flavoenzymes, and FAD and FMN are the most common forms of the flavin prosthetic group. Many of these flavoenzymes have an additive effect on expression. Similar effects of dual promoters on gene expression have been seen in other cases (59).

Acknowledgments—We thank Krishna Madduri, Guido Meurer, Evelyn Wendt-Pienkowski, Bruce Jarvis, Mark Gallo, Sharee Otten, and Heinrich Decke for advice and discussions during the course of this work and Jane Walent and Ronald Niece for the NH2-terminal sequence analysis of tcmG.

REFERENCES

1. Weber, W., Zahner, H., Siebers, J., Schroder, K., and Zeeck, A. (1979) Arch. Microbiol. 121, 111–116
2. Ye, Y., Zhang, H., Xu, S., Zhang, C., and Zai, C. (1984) Antibiot. J. 28, 28–32
3. Eger, E., Noltemeyer, M., Siebers, J., Rohr, J., and Zeeck, A. (1990) J. Antibiot. 43, 1190–1192
4. Drautz, H., Reischenthaler, P., Rohr, H., Rohr, J., and Zeeck, A. (1985) J. Antibiot. 38, 1291–1301
5. Fiedler, H.-P., Rohr, J., and Zeeck, A. (1986) J. Antibiot. 39, 856–859

Fig. 7. Proposed mechanism for the Tcm A2 oxygenase-catalyzed hydroxylation of Tcm A2 to Tcm C involving a monooxygenase (route a) or a dioxygenase (route b).
