Tetracenomycin F2 Cyclase: Intramolecular Aldol Condensation in the Biosynthesis of Tetracenomycin C in *Streptomyces glaucescens*†

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ABSTRACT: Tetracenomycin (Tcm) F2 cyclase, which catalyzes the cyclization of the anthrone Tcm F2 to the naphthacenone Tcm F1 in the biosynthesis of the anthracycline antibiotic Tcm C in *Streptomyces glaucescens*, has been purified to homogeneity and characterized. The N-terminal sequence of the enzyme establishes that it is encoded by the *tcmI* gene, whose deduced product has a molecular weight of 12 728. SDS–PAGE analysis gave a single band with a molecular weight of 12 500, whereas gel-filtration chromatography yielded a molecular weight of 37 500, indicating that the Tcm F2 cyclase is a homotrimer in solution. Under pH $\geq$ 8.0, the enzyme catalyzes the cyclization of Tcm F2 to Tcm F1 and has a K_m of 121 $\pm$ 18.2 μM and V_{max} of 704 $\pm$ 62.3 nmol·min^{-1}·mg^{-1}. In contrast, under pH $\leq$ 6.5, it catalyzes the cyclization of Tcm F2 to 9-decarboxy Tcm F1, a known shunt metabolite of the Tcm C biosynthetic pathway. Tcm F2 cyclase represents the first discrete enzyme for carbon–carbon bond formation via an intramolecular aldol condensation–dehydration mechanism, a key biochemical operation proposed in the early steps of the biosynthesis of all aromatic polyketides.

Polyketides encompass natural compounds with diverse structures including macrolides, polyenes, polyethers, anthracyclines, and tetracyclines, yet apparently they share a common mechanism of biosynthesis: the carbon skeleton of a polyketide is synthesized by sequential condensation of the CoA esters of small fatty acids. This process is catalyzed by a polyketide synthase in the manner that is conceptually similar to the biosynthesis of long-chain fatty acids catalyzed by a fatty acid synthase (Hopwood & Sherman, 1990; Katz & Donadio, 1993). Unlike the latter substances, many polyketides are cyclic compounds whose biosynthesis often involves the formation of one or more six-membered rings. This process is mechanistically the result of one or more intramolecular aldol or Claisen condensations of the oligoketide intermediate, which presumably are catalyzed by the polyketide cyclase as shown in Figure 1.

Little is known about the role that polyketide cyclases play in the cyclization of the oligoketides in vivo, although a number of synthetic oligoketides have been studied in vitro to examine their reactivity and regioselectivity for intramolecular aldol or Claisen condensations (Harris & Harris, 1986). Since different kinds of cyclized compounds such as aklaviketone in *Streptomyces peucetius* (Connors et al., 1990), dehydrorabelomycin in *Streptomyces murayamaensis* (Gould et al., 1992), and tetracenomycin (Tcm)[1] F2 in *Streptomyces glaucescens* (Shen et al., 1993; Yue et al., 1986) formally can be derived from the same class of oligoketide intermediate (Figure 1A–C), a polyketide cyclase could be an important determinant of the structural variation among such cyclized, aromatic polyketides. However, elucidation of the mechanism of these enzymatic aldol or Claisen condensations is challenging because they have no precedents among the well-studied enzymes of primary metabolism.

Molecular genetic analysis of several streptomycetes has recently led to the characterization of genes encoding a number of putative polyketide cyclases. For example, the *actVII* gene for the production of actinorhodin in *Streptomyces coelicolor* (Figure 1D) (Sherman et al., 1991), the *gra–orf4* gene for the production of granaticin in *Streptomyces violaceoruber* (Sherman et al., 1991), the *whiE–orfVI* gene for the production of a spore pigment in *S. coelicolor* (Davis & Chater, 1990) and the *tcmIJN* genes for the production of tetracenomycins in *S. glaucescens* (Figure 1A) (Summers et al., 1992; R. G. Summers, E. Wendt-Pienkowski, H. Motamedi, and C. R. Hutchinson, 1993, unpublished data) have been sequenced and analyzed. These studies have uncovered a highly conserved gene organization but, unfortunately, do not reveal any putative active sites by sequence comparisons; therefore, essentially nothing is known about the reaction mechanism of these enzymatic aldol or Claisen condensations other than the presumption that carbon–carbon bond formation is a separate event from the dehydration (Sherman et al., 1991).

Tcm C, **1**, is an antitumor antibiotic produced by *S. glaucescens* GLA.0 (Weber et al., 1976). We have previously established the biosynthetic pathway of **1** from acetate and malonate with all the biosynthetic intermediates identified (Shen et al., 1993; Yue et al., 1986) and cloned the gene cluster for the biosynthesis of **1** (Motamedi & Hutchinson, 1987). Sequence analysis of the biosynthetic genes (Bibb et al., 1989; Decker & Hutchinson, 1993; Guilfoile & Hutchinson, 1992; Summers et al., 1992; R. G. Summers, E. Wendt-Pienkowski, H. Motamedi, and C. R. Hutchinson, 1993, unpublished data) has provided further support for the proposed biosynthetic pathway to **1** (Figure 1A). From this information, we believe that the naphthacenone Tcm F1, **2**, is biosynthesized by an intramolecular aldol condensation catalyzed by a polyketide cyclase from the corresponding anthrone precursor Tcm F2, **3**. To provide some mechanistic

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[1] Abbreviations: DTT, dithiothreitol; ϵ, molar absorbance index; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; HPLC, high-performance liquid chromatography; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tcm, tetracenomycin; TLC, thin-layer chromatography.

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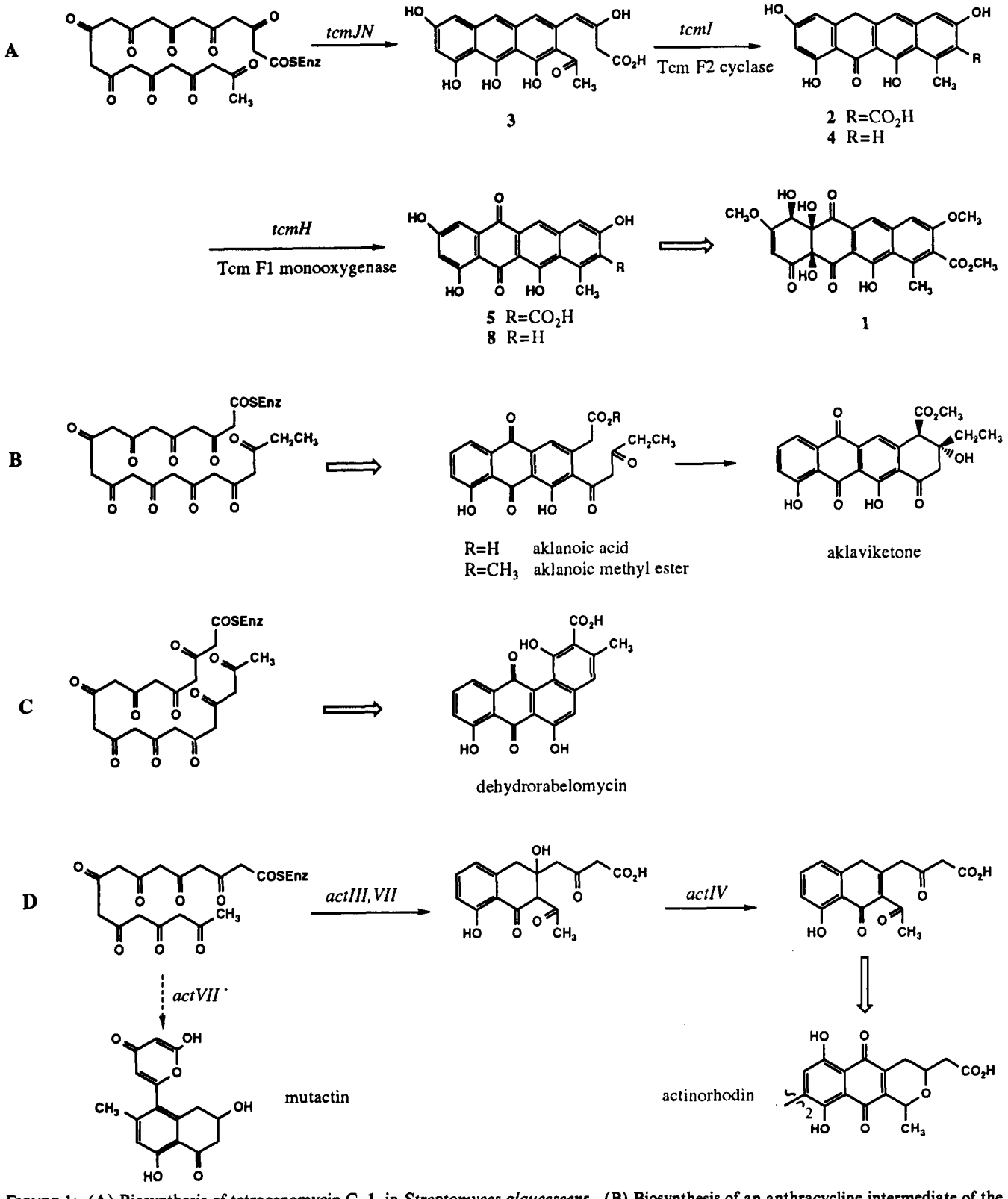

FIGURE 1: (A) Biosynthesis of tetracenomycin C, **1**, in *Streptomyces glaucescens*. (B) Biosynthesis of an anthracycline intermediate of the daunorubicin pathway in *Streptomyces peucetius*. (C) Biosynthesis of a benz[*a*]anthraquinone in *Streptomyces murayamaensis*. (D) Biosynthesis of actinorhodin in *Streptomyces coelicolor*.

detail for a polyketide cyclase-catalyzed intramolecular aldol condensation, we studied the **3** to **2** conversion in vitro and report here the isolation and characterization of the Tcm F2 cyclase from *S. glaucescens* WMH1068 (formerly GLA.11-47) (Motamedi et al., 1986). This enzyme is encoded by the *tcmI* gene and catalyzes the intramolecular cyclization of **3** to **2** or 9-decarboxy Tcm F1, **4**, depending on the pH of the enzymatic reaction.

EXPERIMENTAL PROCEDURES

General. UV/Vis spectra were recorded on a Cary 14/Olis UV/vis spectrophotometer. Refrigerated centrifugation was done in a Sorvall RC-5B superspeed centrifuge. A Pharmacia fast protein liquid chromatography (FPLC) system was used for enzyme purification and all FPLC columns were purchased from Pharmacia LKB. High-performance liquid

chromatography (HPLC) was done with a Waters Model 501 pump system and a Waters 484 variable-wavelength absorbance detector. Enzyme incubations were performed in vessels immersed in a Haake A81 heating/cooling fluid circulator (±0.1 °C). Fermentations of *S. glaucescens* were carried out in a rotary shaker–incubator (New Brunswick Model 25) as described by Summers et al. (1992). Analytic thin-layer chromatography (TLC) was done on precoated Keiselgel 60 F_{254} glass plates (0.25 mm) and was visualized by long- and/or short-wave UV light. Unless specified, common chemicals, biochemicals, and reagents were from commercial sources and were used without further purification.

Protein Analysis. Protein concentrations were determined by the Bradford method with bovine serum albumin as the calibration standard (Spector, 1978). The molecular weight of the enzyme subunit was determined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using the low molecular weight standards of Bio-Rad (phosphorylase *b* 97 400, bovine serum albumin 66 200, ovalbumin 45 000, carbonic anhydrase 31 000, trypsin inhibitor 21 500, and lysozyme 14 400). SDS–PAGE was performed on the PhastSystem (Pharmacia LKB) as described by the manufacturer and gels were stained with Coomassie blue (Heukeshoven & Dernick, 1988). The molecular weight of the native enzyme 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 0.5 mL/min and the column was calibrated with bovine serum albumin (66 000), carbonic anhydrase (29 000), cytochrome *c* (12 400), and aprotinin (6500) as standards (Sigma).

Enzyme Assays. The preparation of **3** and the characterization of **2** and **4** are described elsewhere (Shen et al., 1993).

(*A*) *TLC Method.* This method assays the consumption of **3** and the formation of **2** and/or **4** simultaneously. Typically, 500 μL of assay solution, consisting of 100 μM **3** in 0.1 M Tris/HCl buffer, pH 7.5, in the presence of enzyme (10–50 μL), was incubated at 30 °C. The assay was initiated by addition of **3** and terminated by addition of solid NaH_2PO_4 to saturation and extraction with EtOAC (2 × 250 μL). The EtOAc extracts were collected and concentrated in vacuo to dryness, and then the residue was dissolved in 50 μL of methanol and analyzed by TLC. The TLC plates were developed in $CHCl_3/CH_3OH$/AcOH (85/15/0.25 v/v/v); under these conditions, **3**, **2**, and **4** have R_f values of 0.57, 0.38, and 0.86 and, under UV light, display characteristic blue, yellow, and yellow fluorescence, respectively. This assay method was used throughout the enzyme purification.

(*B*) *HPLC Method.* This method provides a quantitative analysis of the consumption of **3** and the formation of **2** and/or **4**. A typical assay solution of 200 μL consists of 100 μM **3** in 0.1 mM Tris/HCl buffer, pH ranged from 6.5 to 8.5, or in 0.1 M Bis-Tris/HCl buffer, pH ranged from 5.5 to 7.5, in the presence of 1.4 μg of the Tcm F2 cyclase. The assay solution was preincubated at 30 °C for 10 min; the reaction then was initiated by addition of **3** and terminated after a 5-min incubation at 30 °C by addition of solid NaH_2PO_4 to saturation and extraction with EtOAc (200 μL). The EtOAc extract was analyzed by HPLC with **1** as an internal standard on a Nova-Pak C_{18} column developed with a linear gradient from CH_3CN/H_2O/AcOH (20/80/0.1% v/v/v) to CH_3CN in 10 min at a flow rate of 2 mL/min with UV detection at 280 nm; under these conditions **3**, **1**, **2**, and **4** have retention times of 5.1, 6.7, 7.3, and 8.6 min, respectively. This analysis was used for the determination of the pH dependence of the Tcm F2 cyclase.

(*C*) *Spectrophotometric Method.* The production of **2** can be continuously monitored by measuring the absorbance at 426 nm, at which in 0.1 M Tris/HCl, pH 8.0, $\epsilon_{426\ nm}$ for **3** and **2** are approximately 3700 and 17 500 $M^{-1}\ cm^{-1}$, respectively. A typical assay solution of 800 μL consists of **3**, ranged from 19 to 150 μM in 0.1 M Tris/HCl buffer, pH 8.0, in the presence of 2.8 μg of the Tcm F2 cyclase. The assay solution was preincubated in the cuvette for 10 min and the reaction was initiated by addition of **3**. The production of **2** was continously monitored by measuring the increase of the absorbance at 426 nm, from which the initial velocity of the reaction was determined after the reaction reached the steady state. This assay method was used for the determination of the kinetic parameters.

Enzyme Purification. All steps were carried out at 4 °C.

Step 1: Preparation of Cell-Free Extract. Cultures of *S. glaucescens* WMH1068 were grown in R2YENG medium (Motamedi et al., 1986) in a 2-L baffled Erlenmeyer flask. After incubation at 30 °C and 300 rpm for 28 h, cells were harvested by centrifugation (13600*g*, 20 min, 4 °C) and washed sequentially with 0.5 M NaCl and 0.1 M sodium phosphate buffer, pH 7.2, with centrifugation as necessary. The washed cells were suspended in 100 mM sodium phosphate buffer (pH 7.2)–0.1 mM phenylmethanesulfonyl fluoride–1 mM DTT–1 mM EDTA–10% glycerol (10 mL/g of cells). Lysozyme (1 mg/mL) was added and the mixutre was left to incubate at room temperature for 2 h. To this viscous slurry, solid $MgCl_2$ (5 mg/mL) and DNase (1 μg/mL) were added. The resulting slurry was incubated on ice for 1 h and cell debris was removed by centrifugation (27500*g*, 20 min, 4 °C) to yield a cell-free extract.

Step 2: Ammonium Sulfate Fractionation. The cell-free extract was brought to 41% saturation (234 g/L) by addition of solid ammonium sulfate. The suspension was stirred for 1 h and centrifuged as above to remove the precipitate. The resulting supernatant was brought to 62% saturation (375 g/L) with solid ammonium sulfate and stirred for an additional 1 h. Centrifugation as above afforded a pellet that had the enzyme activity.

Step 3: Sephacryl S-200 Column. The ammonium sulfate pellet was dissolved in a minimum volume of 20 mM sodium phosphate buffer (pH 7.2)–150 mM NaCl–1 mM DTT and applied to a Sephacryl S-200 HR column (2.6 × 60 cm). Fractions (2.0 mL/min, 5-mL fractions) were collected after elution with the same buffer.

Step 4: Mono Q HR 10/10 Column. Fractions containing the enzyme activity after gel-filtration chromatography were dialyzed against 25 mM Tris/HCl buffer (pH 8.0)–1 mM DTT and then applied to a mono Q HR 10/10 column. The column was washed with 25 mM Tris-HCl buffer (pH 8.0)–1 mM DTT and then developed with a linear 100-mL gradient from 0 to 0.6 M NaCl in the same buffer (2 mL/min, 2-mL fractions).

Step 5: Phenyl-Superose HR 5/5 Column. The active fractions after anion-exchange chromatography were brought to 1.0 M ammonium sulfate by addition of solid ammonium sulfate and were then applied to a phenyl-Superose HR 5/5 column. The column was washed with 50 mM sodium phosphate (pH 7.2)–1 mM DTT–1.0 M $(NH_4)_2SO_4$ and then developed with a linear 15-mL gradient from 1.0 to 0 M ammonium sulfate in the same buffer (0.5 mL/min, 0.5-mL fractions).

Table I: Purification of Tcm F2 Cyclase from *S. glaucescens* WMH1068

step	protein (mg)	activity × 10^3 (nmol·min^{-1})	yield (%)	specific activity (nmol·min^{-1}·mg^{-1})	purification (*x*-fold)
cell-free extract	2.82×10^3	1.61	100	0.571	1.00
$(NH_4)_2SO_4$ precipitation	1.20×10^3	1.21	74.9	1.01	1.87
Sephacryl S-200 HR	242	0.355	22.0	1.46	2.56
mono-Q HR 10/10	4.67	0.345	21.4	73.9	130
phenyl-Superose HR 5/5	0.915	0.225	13.9	245	429
Superose 6 HR 10/30	0.414	0.128	7.94	309	541

Step 6: Superose 6 HR 10/30 Column. The active fractions after hydrophobic chromatography were concentrated with a Centricon 10 (Amicon) and applied to a Superose 6 HR 10/30 column (a maximum of 200 μL was loaded per run). It was then eluted with 20 mM sodium phosphate buffer (pH 7.2)–150 mM NaCl–1 mM DTT (0.5 mL/min, 0.5-mL fractions) to give the final preparation of the Tcm F2 cyclase.

N-Terminal Sequence Determination. The amino-terminal sequence of the purified Tcm F2 cyclase was determined by automated Edman degradation after desalting by passage through a Vydac protein C_4 reverse-phase HPLC column developed with a linear 50-mL gradient from 5% to 100% acetonitrile in 0.1% (v/v) trifluoroacetic acid (1.0 mL/min, 1.0-mL fractions).

RESULTS

Purification of the Tcm F2 Cyclase from S. glaucescens WMH1068. The Tcm F2 cyclase is copurified with the Tcm F1 monooxygenase from *S. glaucescens* WMH1068, which catalyzes the oxidation of **2** to Tcm D3, **5** (Shen & Hutchinson, 1993), until the Sephacryl S-200 chromatography and is readily separated from the latter enzyme upon anion-exchange chromatography on the mono Q column. Therefore, in the early stage of the purification, **5** was often the product that resulted from the assay of the Tcm F2 cyclase with **3** as substrate. To minimize this problem, the Tcm F2 cyclase activity was precipitated with $(NH_4)_2SO_4$ at 41–62% saturation from a cell-free extract of *S. glaucescens* WMH1068. The active pellet was first subjected to size-exclusion chromatography on the Sephacryl S-200 column. However, the Tcm F2 cyclase was eluted as a very broad peak of activity, resulting in a major loss of enzyme activity in the overall purification process. This chromatographic step proved later to be necessary, however, because it removed many of the contaminating proteins so that the Tcm F2 cyclase could be resolved as a very sharp activity peak by the subsequent anion-exchange chromatography. Tcm F2 cyclase is a very hydrophobic protein; it bound to the phenyl-Superose column tightly and was eluted with approximately 0 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate (pH 7.2)–1 mM DTT. After the above three chromatographic steps, the cyclase was finally resolved by a second size-exclusion chromatography on the Superose 6 column and the pure enzyme was eluted as a symmetric sharp peak. As summarized in Table I, these procedures gave Tcm F2 cyclase with an overall 541-fold purification.

The purified enzyme was homogeneous when examined by SDS–PAGE where it migrated as a single band (Figure 2) with a relative subunit molecular weight of 12 500.

N-Terminal Sequence Determination. The purified Tcm F2 cyclase (85 pmol) was subjected to amino-terminal seuqencing and the first 14 residues were determined as A-Y-R-A-L-M-V-L-R-M-D-P-A-D. These data establish that Tcm F2 cyclase is encoded by the *tcmI* gene that has been sequenced recently (R. G. Summers, E. Wendt-Pienkowski, H. Motamedi, and C. R. Hutchinson, 1993, unpublished data); Figure 3 shows the deduced sequence of the *tcmI* gene product. We presume that the fMet is cleaved posttranslationally since the amino terminus of the isolated enzyme was found to be Ala. From the DNA sequence of the *tcmI* gene, it can be calculated that the Tcm F2 cyclase has a molecular weight of 12 728.

FIGURE 2: SDS–PAGE analysis of Tcm F2 cyclase on an 8–25% gradient PhastGel. Lane 1, Bio-Rad low molecular weight standards described under Experimental Procedures; lane 2, Tcm F2 cyclase.

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  1 MAYRALMVLRMDPADAEHVAAAFAEHDTTELPLEIGVRRRVLFRFHDLYM
 51 HLIEADDDIMERLYQARSHPLFQEVNERVGQYLTPYAQDWEELKDSKAEV
101 FYSWTAPDS
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FIGURE 3: Amino acid sequence of Tcm F2 cyclase deduced from the *tcmI* gene.

Molecular Weight Determination. As determined by chromatography on a Superose 6 HR 10/30 column, the native form of Tcm F2 cyclase has a molecular weight of 37 500.

pH Dependence. The effect of pH on the Tcm F2 cyclase is shown in Figure 4. Although the cyclase displays an optimal pH of 6.0–6.5 for the conversion of **3**, only a very small dependence of the enzyme activity on pH in the range from 5 to 8.5 was observed. However, the cyclase causes **3** to cyclize into two different products depending on the pH of the reaction solution: at a pH ≥ 8.0, **3** is cyclized to give **2**, but **4** becomes the predominant product if the same reaction is performed at a pH ≤ 6.5. Pure **2** was incubated under the identical conditions in either 0.1 M Tris/HCl, pH 8.0, or Bis-Tris/HCl, pH 6.5, for 5–10 min and no change in the amount of **2** was observed upon HPLC analysis of the reaction mixture. This eliminates the possibility that **4** could have resulted from decarboxylation of **2** [chemical precedents (Harris & Harris, 1986) make it unlikely that decarboxy-**3** would have cyclized to **4**].

Kinetics. Kinetic analysis was carried out on the basis of a first-order treatment with a steady-state approach. After a initial delay (up to about 5 μM conversion of **3**), where the formation of **2** displays a nonlinear relationship vs time, the reaction reaches its steady state. The effect of the initial

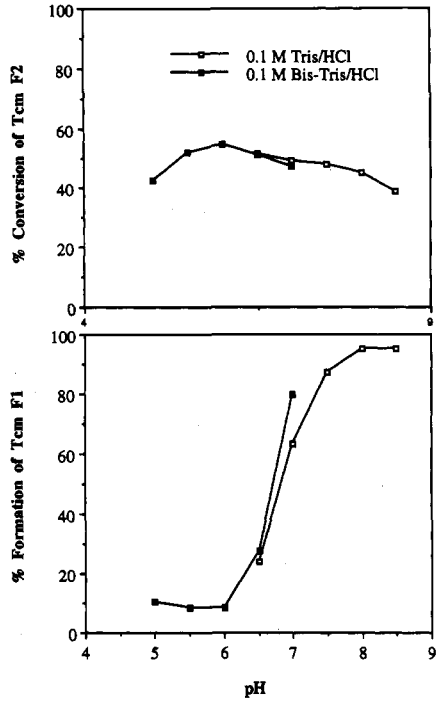

FIGURE 4: Effect of pH on Tcm F2 cycalse activity and on the product distribution of the enyzmatic cyclization of 3 in 0.1 M Tris/HCl or 0.1 M Bis-Tris/HCl buffers.

concentration of 3 on the formation of 2 in the steady state indicates that Tcm F2 cyclase follows Michaelis–Menten kinetics. Velocities were then fitted to the Michaelis–Menten equation by using Brooks' enzyme kinetic software (Brooks, 1992), and from a nonlinear regression analysis using Marquardt–Levenberg algorithms, the apparent K_m and V_{max} were determined to be 121 ± 18.2 μM and 704 ± 62.3 nmol·min^{-1}·mg^{-1}, resectively.

DISCUSSION

The Tcm F2 cyclase from *S. glaucescens* WMH1068 has been purified to homogeneity and its N-terminal sequence establishes that it is encoded by the *tcmI* gene. Although genetic analysis of several streptomycetes has led to the characterization of genes encoding a number of putative polyketide cyclases, including the TcmI protein (Davis & Chater, 1990; Sherman et al., 1991; Summers et al., 1992; R. G. Summers, E. Wendt-Pienkowski, H. Motamedi, and C. R. Hutchinson, 1993, unpublished data), the purification and characterization of the Tcm F2 cyclase supports these predictions with direct enzymological evidence about an authentic polyketide cyclase. SDS–PAGE analysis shows that the purified enzyme displays a single band with a molecular weight of 12 500, in good agreement with the value of 12 728 (excluding the fMet) predicted from translation of the *tcmI* gene. The molecular weight of the native enzyme, 37 500, suggests that the Tcm F2 cyclase is a homotrimer in solution.

Tcm F2 cyclase catalyzes the cyclization of an anthrone 3 to the naphthacenone 2, requiring no cofactor. This reaction represents the first discrete enzyme for carbon–carbon bond formation via an intramolecular aldol condensation–dehydration mechanism, a key biochemical operation proposed in the early steps of the biosynthesis of all aromatic polyketides. [6-Methylsalicyclic acid (Beck et al., 1990) and chalcone (Schuz et al., 1983) synthases also must catalyze the respective aldol and Claisen condensations that produce aromatic rings, but the responsible domain or active site has not been identified

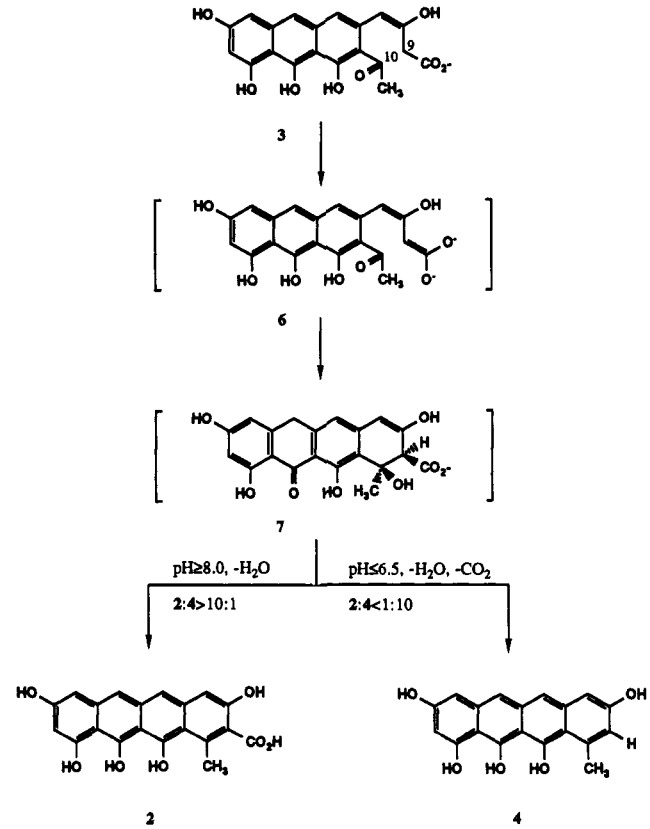

FIGURE 5: Tcm F2 cyclase-catalyzed cyclization of 3 to 2 and 4 in 0.1 M Tris/HCl or Bis-Tris/HCl buffers.

in these polyketide synthases.] Formally, the dehydroquinate synthase catalyzed conversion of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate to dehydroquinate (Bender et al., 1989a,b; Widlanski et al., 1989) and the naphthoate synthase-catalyzed conversion of *O*-succinylbenzoic acid CoA ester to 1,4-dihydroxy-2-naphthoic acid (Bryant & Bentley, 1976; Heide et al., 1982; Meganathan & Bentley, 1979) bear some resemblance to the Tcm F2 cyclase-catalyzed conversion of 3 to 2. However, the former reactions differ mechanistically from the latter in most aspects: dehydroquinate synthase is a multifunctional enzyme mediating five transformations and contains 1 mol each of tightly bound Co(II) and NAD$^+$, and naphthoate synthase, which has only been detected in the form of a cell-free extract, catalyzes the condensation between a methylene group and an activated (as CoA ester) carboxyl group, essentially a Claisen reaction.

From the organic chemistry of intramolecular aldol condensations and the pH dependence of Tcm F2 cyclase, the enzymatic cyclization of 3 can be considered to occur in two steps. Since spontaneous cyclization was not observed in the absence of the enzyme under the conditions tested, this seemingly facile reaction requires enzyme catalysis. We propose that in the first step upon addition of Tcm F2 cyclase an enzyme-bound C-9 enolate, 6, is formed that attacks the C-10 carbonyl group to yield the intramolecular aldol adduct 7 (Figure 5). Intermediate 7 could then aromatize into 2 or 4 in the second step, depending on the conditions of the reaction. At pH ≥ 8.0, the OH group at C-10 would be a poor leaving group, and therefore, the dehydration step is likely to be initiated by deprotonation at C-9, presumably by a basic residue of the enzyme, to give 2 as the product. In contrast, at pH ≤ 6.5, the ability of the enzyme to deprotonate C-9 could be diminished by protonation of the basic residue of the enzyme, while protonation of the C-10 OH group would make

it an excellent leaving group. Consequently, facile dehydration coincident with decarboxylation results in the production of **4**. The latter result provides an explanation for the isolation of **4** and its corresponding oxidized product Tcm D1, **8**, in the form of shunt metabolites from the fermentation of *S. glaucescens* GLA.0 (Yue et al., 1986).

Other polyketide cyclases that are predicted to have the same catalytic mechanism as Tcm F2 cyclase also are thought to operate in a stepwise manner. For instance, the isolation of mutactin (Figure 1D) from an *S. coelicolor actVII* mutant (Zhang et al., 1990) and the suggestion (Bartel et al., 1990) that *actIV* mutants accumulate the bicyclic product of aldol condensation directed by the *actVII* gene (Figure 1D), which in the *actIV*$^+$ background would undergo the enzyme-mediated dehydration shown, have been offered as support for this idea. This indirect evidence is helpful in formulating ideas about the nature of such enzymes, prior to their actual purification. On the other hand, the lack of significant sequence similarity between the Tcm F2 cyclase and the deduced products of the *actVII* and *actIV* genes suggests that the topology of the active sites among these proteins, and perhaps even their catalytic mechanisms, could be quite different.

It is interesting that the organization of the *tcmHI* genes (R. G. Summers, E. Wendt-Pienkowski, H. Motamedi, and C. R. Hutchinson, 1993, unpublished data) suggests that expression of these two genes is translationally coupled and thus that their proteins might be produced in stoichiometric amounts in vivo. We have previously reported (Shen & Hutchinson, 1993) that the *tcmH* gene encodes the Tcm F1 monooxygenase that catlayzes the oxidation of **2** to **5**. It has a molecular weight of 12 554 and exists as a homotrimer in solution with an apparent K_m of 7.47 μM and V_{max} of 473 nmol·min^{-1}·mg^{-1}. Remarkably, the Tcm F2 cyclase has a molecular weight of 12 728 and is a homotrimer in solution as well, with an apparent K_m of 121 μM and V_{max} of 704 nmol·min^{-1}·mg^{-1}. Since the V_{max}/K_m for the former enzyme (0.063) is approximately 10 times larger than that of the latter (0.0057), the intrinsic nature of these two consecutively acting enzymes explains why none of the tetracenomycin-producing strains appears to accumulate **2**.

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