Reconstitution of the Iterative Type II Polyketide Synthase for Tetracenomycin F2 Biosynthesis†

Wuli Bao,‡ Evelyn Wendt-Pienkowski,‡ and C. Richard Hutchinson*,‡,§

School of Pharmacy and Department of Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

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ABSTRACT: The tetracenomycin polyketide synthase (TCM PKS), a type II complex, produces TCM F2, a precursor of TCM C in *Streptomyces glaucescens*, and consists of at least the TcmK, -L, -M, and -N proteins. The TcmK/TcmL ketosynthase subunits were purified from overexpression of their genes in *Streptomyces lividans*. TcmK (calculated molecular mass 45 kd) and TcmL (calculated molecular mass 42 kd) function as a heterodimeric $\alpha\beta$ complex based on observing that only the purified complex complemented TCM PKS activity in protein extracts made from strains bearing *tcmK* or *tcmL* deletion mutants to make TCM F2 in vitro, and that the molecular mass of the purified complex was 90 kd as estimated by gel filtration chromatography. The TCM PKS activity was reconstituted with purified protein components, indicating that the minimal set of proteins required to make TCM F2 included the ketosynthase complex (TcmKL), an acyl carrier protein (TcmM), a malonyl CoA:ACP acyltransferase (MAT), and a cyclase (TcmN). The MAT was required to catalyze the transacylation between malonyl-CoA and TcmM, although a relatively slow spontaneous transacylation also occurred in a reaction without the MAT. Acetyl-CoA, the proposed starter unit for the TCM PKS, was not required for the production of TCM F2 in vitro, although it could be incorporated into this polyketide to a small extent. TcmJ, a PKS protein without a known function, greatly increased the production of TCM F2 but could not replace TcmN as a cyclase in the reconstituted system.

Polyketide synthases (PKSs)[1] catalyze the biosynthesis of a large group of natural products, many of which are medically important or have other pharmacological activities (*1*, *2*). They have been classified as type I PKSs, which are large multifunctional proteins with distinctive active sites for every enzyme-catalyzed step (*3*, *4*); and type II PKSs, which have a separate polypeptide for each activity, use each active site iteratively, and typically produce polycyclic aromatic compounds (*2*). In both types, the PKS catalyzes the sequential condensation of carboxylate units to a growing carbon chain in a manner that is similar to the biosynthesis of fatty acids by fatty acid synthases (FAS) (*1*−*4*). Interest in understanding the biosynthesis of the polyketides has risen because of the potential for producing new polyketide-derived drugs by genetically engineered PKSs in recombinant microorganisms (*5*, *6*).

Our knowledge about polyketide biosynthesis has advanced rapidly with the advent of studies of the molecular genetics of the *Streptomyces* and related genera over the past 15 years. Clusters of genes that govern this process have been identified, cloned, and characterized functionally (*1*, *2*). Considerable interest has been directed into analyzing the biosynthesis of the polyketide framework of natural products, because this stage of the pathway determines the size and most of the chemical functionality of the final product (*1*, *2*). For tetracenomycin (TCM) C biosynthesis in *Streptomyces glaucescens*, the *tcmJ*, *tcmK*, *tcmL*, *tcmM*, and *tcmN* genes encode the PKS responsible for the formation of the carbon skeleton (*7*, *8*); the *actI-Orf1*, *-2*, and *-3* genes plus *actIII*, *actVII*, and *actIV* perform this role in the actinorhodin (ACT) pathway in *Streptomyces coelicolor* (*9*, *10*). Biochemical characterization of most of the component enzymes has been carried out in vivo by mutation of their genes to identify the accumulated products (*1*, *2*) and by combinatorial expression to identify their catalytic functions (*3*−*6*). Yet a detailed understanding of the mechanism and the properties of these enzymes cannot come from genetic studies alone.

Cell-free studies of the enzymology of type II PKSs have been successfully used to investigate the biosynthesis of TCM F2, a precursor of TCM C (*11*, *12*), and the ACT precursors (*13*). Carreras and Khosla (*14*) recently demonstrated that the ACT ketosynthase (KS_α) and the chain length factor (which we call KS_β here) can be purified as a tetrameric complex (presumably $\alpha_2\beta_2$) and together with the ACT acyl carrier protein (ACP) and a malonyl-CoA:ACP acyltransferase (MAT) could be used to produce polyketides in vitro. The MAT, which is believed to be a component of an *S. coelicolor* FAS encoded by the *fabD*, *fabH*, *fabC*, *and*

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* Correspondence should be addressed to this author at the School of Pharmacy, University of Wisconsin, 425 N. Charter St., Madison, WI 53706. Fax: 608-262-3134. E-mail: crhutchi@facstaff.wisc.edu.

‡ School of Pharmacy.

§ Department of Bacteriology.

[1] Abbreviations: ACP, acyl carrier protein; CoA, coenzyme A; cpm, counts per minute; DTT, dithiothreitol; FAS, fatty acid synthase; IPTG, isopropyl β-D-thiogalactopyranoside; kd, kilodaltons; MAT, malonyl-coenzyme A:ACP acyltransferase; PAGE, polyacrylamide gel electrophoresis; PKS, polyketide synthase; TCA, trichloroacetic acid; TCM, tetracenomycin; SDS, sodium dodecyl sulfate.

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fabB genes (*15*), was absolutely required (*14*), even though Hitchman et al. (*16*) have reported that a MAT is not required for the acylation of many type II PKS ACPs because these proteins, unlike typical FAS ACPs, are capable of self-acylation (malonylation). Nonetheless, the minimal set of proteins needed to reconstitute the synthesis of ACT precursors in vitro include the tetrameric KS $\alpha_2\beta_2$ complex and the ACP encoded by the three *actI* genes and the *fabD* MAT (*14*).

TCM C biosynthesis is one of the current models for a type II PKS. Development of the cell-free biosynthesis of TCM F2 (*11*) facilitated the purification and reconstitution of the individual components of the TCM PKS. By using the protein extract from a recombinant strain, it was shown that to make TCM F2 in vitro four genes encoding two KS subunits (TcmK[α]/TcmL[β]), an ACP (TcmM), and a cyclase (TcmN) had to be expressed together in a TCM PKS-free *S. glaucescens* mutant (*11*). Inclusion of the *tcmJ* gene greatly increased the amount of TCM F2. TcmM had previously been purified and characterized by its ability to accept malonate in the MAT assay (*8*), and the bifunctional TcmN cyclase/*O*-methyltransferase was subsequently purified and studied through its ability to catalyze the *O*-methylation of TCM D3 to TCM B3 (*12*). Addition of TcmN purified from *Escherichia coli* or a *Streptomyces* sp. to the protein extract containing the TcmK, TcmL, and TcmM enzymes restored the production of TCM F2 (*12*). In the absence of TcmN, this crude protein extract produced a limited amount of TCM F2 and other shunt compounds resulting from the spontaneous cyclization of the TCM decaketide.

The presumed function of the TcmK and TcmL KS subunits is based on the high sequence similarity to their counterparts in bacterial FASs (*17*). Purification of these subunits for reconstitution of the TCM PKS in vitro had to be done to enable elucidation of the PKS mechanism; however, a possible requirement for other protein components to form TCM F2, some of which might not have been identified in the genetic studies, increased the difficulty of enzyme purification and in vitro reconstitution. Nevertheless, by employing an enzyme assay based on recombinant strains lacking either TcmK or TcmL, we have been able to purify the TcmK/TcmL complex and then reconstitute the Tcm PKS activity with the purified components, thereby establishing the minimal number of enzymes required to make TCM F2. We also report that the *S. glaucescens fabD* MAT (*18*) is absolutely required but that acetyl-CoA, the presumed starter unit for assembly of the TCM decaketide, is not required in vitro.

EXPERIMENTAL PROCEDURES

Chemicals, Bacterial Strains, and Plasmid Constructs. [^{14}C]Malonyl-CoA (54 mCi/mmol) was obtained from Amersham Life Science (England), and [^{14}C]acetyl-CoA (18 Ci/mmol) was from ICN Radiochemicals (USA). All other chemicals were from Sigma Chemical Co. (USA). The *tcmN* and *tcmM* expression plasmids were from previous work (*8*, *12*). The *E. coli* BL21 strain was purchased from Stratagene (USA), and the *Streptomyces lividans* 1326 strain was obtained originally from David Hopwood (England).

Assay and Purification of TcmM and the fabD MAT. The TcmM ACP was assayed for its ability to serve as an acceptor of malonate from malonyl-CoA catalyzed by a MAT (*8*). The *tcmM* expression plasmid, a variation of pWHM701 (*8*) in which the *melC1* promoter was replaced with the *ermE*p* promoter by cloning an approximately 300 nt *Kpn*I−*Sph*I segment containing *ermE*p* into the *Kpn*I and *Sph*I sites of pWHM701, was introduced into *S. lividans* by transformation, and the recombinant strain was grown in R2YE media (*19*) containing thiostrepton (10 μg/mL). Assay and purification of holo-TcmM were then carried out as described (*8*).

The *fabD* MAT was purified from *E. coli*. A plasmid for expressing *fabD* under control of a phage T7 promoter (pLH16, obtained from K. Reynolds) was introduced into *E. coli* BL21 by standard means (*20*), and the recombinant strain was grown at 37 °C in LB medium (*20*) with ampicillin (50 μg/mL) overnight. When the culture reached an A_{600} of 0.6, IPTG was added (0.5 mM) and the culture grown for another 4 h. Cells were harvested and proteins were extracted as described by Shen et al. (*8*). The crude proteins (5 mL) in 20 mM Tris-HCl buffer (pH 8.0, 10% glycerol, 0.1 M NaCl) were loaded onto a Sephacryl S-200 fast protein liquid chromatography (FPLC) column (Pharmacia) and eluted with the same buffer at 1 mL/min. The fractions with MAT activity [assayed as described below by using *E. coli* FAS ACP (Sigma) as the acceptor of radioactive malonate] were pooled and loaded onto a MonoQ FPLC column (Pharmacia) and eluted with a gradient of the same buffer containing NaCl from 0.1 to 0.6 M. The fractions with MAT activity (the major peak) were pooled and concentrated with a Centriprep-10 (Millipore), and the purity was examined by SDS−PAGE as described (*8*, *18*) (Figure 1).

A typical MAT assay solution contained 20 mM sodium phosphate (pH 7.2), 2 mM DTT, 500 nM MAT, and the ACP (5 μM) in a final volume of 100 μL. The assay was initiated by addition of 2 μM [2-^{14}C]malonyl-CoA (10 000 cpm) in 20 mM phosphoric acid (pH 3.5), kept at room temperature for 1 min, and terminated by the addition of 100 μL of bovine serum albumin solution (10 mg/mL) and 400 μL of a 20% (v/v) trichloroacetic acid (TCA) solution. Precipitated proteins were centrifuged, and the pellet was washed with a 10% TCA solution. The pellet was dissolved in 1 M Tris base, and the transacylation activity was determined by liquid scintillation counting.

Plasmid Construction, Assay, and Purification of the TcmK/TcmL KS Subunits. Before TcmK and TcmL were purified, mutant strains were constructed with complementary deletions in the TCM PKS genes. Plasmid pWHM722 (*11*), which contains the *tcmKLMN* genes expressed under control of the strong, constitutive *ermEp** promoter, was digested with *Pvu*I to remove a 915 base pair (bp) segment inside *tcmK* and then religated. The resulting plasmid was digested with *Bcl*I and *Xba*I to remove the 4.3 kbp segment containing part of the *tcmK* and the *tcmLMN* genes, and this segment was cloned into *Eco*RI- and *Xba*I-digested pWHM3, an *E. coli*−*Streptomyces* shuttle vector (*21*), along with an 800 bp *Eco*RI−*Bcl*I DNA segment from pWHM732 (*7*) containing *ermEp** and *tcmJ*. The resulting pWHM1001 plasmid contained the *tcmJ*Δ*KLMN* genes expressed under control of *ermEp** and a thiostrepton resistance gene. The *tcmJK*Δ*LMN* expression vector (pWHM1002) was constructed from pWHM722 in the same manner by removing a 969 bp *Sca*I−*Nru*I segment inside *tcmL*.

For overexpression of the *tcmKL* genes in *Streptomyces* sp., a 3025 bp *Kpn*I−*Mro*I segment containing *tcmKL* and

promoter *ermEp** was isolated from pWHM722 and ligated into pGEM-7Zf (Promega). The 3045 bp *Eco*RI−*Nsi*I fragment from the resulting plasmid was ligated into pWHM721 (*11*) to form plasmid pWHM1003. The same fragment was used to construct the [his$_6$]-tagged *tcmK*/*tcmL* expression plasmid pWHM1004 by ligating the fragment into pWHM3, then removing a 135 bp *Bcl*I−*Pst*I fragment containing part of *tcmJ* and part of *tcmK* from the resulting plasmid, and replacing it by a 102 bp linker. The linker consisted of part of *tcmK*, a [his$_6$] sequence whose codon usage was optimized for *Streptomyces* sp. (U. Roos and C.R.H., unpublished work), and a ribosome binding site.

Freshly transformed *S. lividans* 1326 strains containing any one of the plasmid constructs described above were first grown in 5 mL of R2YE medium containing thiostrepton (20 μg/mL) in a rotary shaker (300 rpm, 30 °C) for 48 h. All the 5 mL culture was inoculated into 50 mL of R2YE medium and grown under the same conditions for 28 h. The mycelial cells were pelleted by centrifugation (8000*g*, 10 min, 4 °C) and dissolved in 20 mL of glycerol (20%, v/v) as seed and stored at −80 °C. A 5 mL portion of the seed was used to inoculate 1.2 L of R2YE medium containing thiostrepton (10 μg/mL) in a 2.5 L stirred fermenter (New Brunswick BioFlo IIc) and grown (30 °C, stirring rate of 400 rpm, air flow rate of 1.5 L/min) for 30 h with automatic addition of antifoam B (1%, v/v, Sigma) control. Cells were harvested by centrifugation (8000*g*, 10 min, 4 °C) and washed with 10% sucrose (w/v), and then used to prepare a cell extract as described earlier (*11*). Ammonium sulfate (504 g/L) was added to the cell extract, and the precipitate was collected by centrifugation (25420*g*, 20 min, 4 °C). The resulting pellet was dissolved in 100 mM sodium phosphate (pH 7.2), 2 mM DTT, and 10% glycerol (v/v) (buffer A) and, using a PD-10 column (Pharmacia), desalted into buffer A at a concentration of 2.5 mg/mL.

The complete TCM PKS assay solution (250 μL) contained 150 μM [2-^{14}C]malonyl-CoA (10 000 cpm), 2 mM DTT, 20 μL of TcmK/TcmL crude extract or fractions from purification chromatography, and 50 μL (0.5 mg) of protein from the *tcmJ*Δ*KLMN* or *tcmJK*Δ*LMN* strains in 0.1 M sodium phosphate buffer (pH 7.5). The assays were initiated by addition of malonyl-CoA, incubated at 30 °C for 60 min, and terminated by addition of 150 mg of NaH_2PO_4. The products were extracted with 2 × 400 μL of ethyl acetate, and the combined extracts were dried under vacuum in Eppendorf tubes. After addition of acetonitrile (10 μL) to the tube, the solution was subjected to analysis by high-performance liquid chromatography (HPLC) on a Waters Radi-Pak C_{18} column (5 mm, 8 × 100 mm). The column was run with a linear gradient of acetonitrile/H_2O/glacial acetic acid from 20:80:0.1 to 60:40:0.1 (v/v) in 10 min at 2 mL/min. TCM F2 was eluted with a retention time of 7.8 min.

To purify the TcmK/TcmL proteins, *S. lividans* containing pWHM1003 was grown and the cells were harvested as described above. The cells were lysed in buffer A without glycerol as described (*11*) and centrifuged (25420*g*, 20 min, 4 °C) to obtain a crude cell-free extract. Ammonium sulfate (226 g/L) was dissolved into the crude extract, and the solution was centrifuged (25420*g*, 20 min, 4 °C). The supernatant was applied to a Phenyl Superose CL-4 FPLC column (Pharmacia) and washed with buffer A containing 1.7 M ammonium sulfate and no glycerol. The TcmKL activity was eluted with a linear 200 mL gradient from 1.7 to 0 M ammonium sulfate in buffer A with no glycerol at a flow rate of 2.5 mL/min. After the gradient, the column was eluted with 200 mL of buffer A without glycerol, and then 15% (v/v) glycerol was added to the fractions exhibiting TcmKL activity. Fractions containing this activity were pooled, concentrated in a centrifuge by an ultrafiltration device (Centriprep-30, Amicon), and subjected to gel filtration by FPLC on a Sephacryl S-200 chromatography column (Pharmacia) in buffer A with 0.1 M NaCl. The column was eluted at 1.0 mL/min, and 2 mL fractions were collected. Fractions with TcmKL activity were pooled, and the proteins therein were bound to a DEAE BioGel A agarose (BioRad) column. TcmKL activity was eluted with a 200 mL gradient of NaCl from 0.1 to 0.6 M in buffer A. The fractions with TcmKL activity were pooled, concentrated by Centriprep-30, and, using a PD-10 column, desalted into buffer A at a concentration of approximately 1 mg/mL.

The partially purified TcmK and TcmL proteins were analyzed by gel filtration chromatography to determine the size of the protein complex (Figure 2) and by SDS−PAGE (3 μg, 10−20% gradient gel, BioRad Ready Gel) (Figure 1) to determine the purity. A concentrated protein sample (5 mg, 2 mL) of TcmKL was loaded onto a Sephacryl S-200 column (2.6 × 60 cm) with buffer A containing 0.1 M NaCl and eluted with the same buffer at 1 mL/min. Carbonic anhydrase (29 kd), bovine serum albumin (66 kd), alcohol dehydrogenase (150 kd), and β-amylase (200 kd) were used to establish the standard curve using the partition coefficient K_p (*22*).

To purify [his$_6$]-tagged-TcmK/TcmL, *S. lividans* containing pWHM1004 was used in the procedures described above. After gel filtration chromatography, the [his$_6$]-TcmKL activity was bound to a nickel affinity column (Novagen) in buffer A without DTT. A step gradient of imidazole from 0.2 to 1 M was applied to the column to elute the [his$_6$]-TcmKL activity. Fractions with the desired activity were concentrated with a Centriprep-30, the eluting buffer was replaced with buffer A using a PD-10 column, and the resulting solution was analyzed on SDS−PAGE (10−20% gradient gel) (Figure 1) and used in the reconstitution of TCM PKS activity.

In Vitro Reconstitution of TCM PKS Activity. The complete reaction solution (250 μL) consisted of 150 μM [2-^{14}C]malonyl-CoA (16 000 cpm), 2 mM DTT, 5 μM ACP, 2 μM TcmN, 2 μM TcmKL complex, and 1 μM MAT in 0.1 M phosphate buffer (pH 7.2). TcmM (1 mg/mL) was purified as described (*8*), and TcmN (2.5 mg/mL) was purified from *E. coli* as described by Shen and Hutchinson (*12*). To study the function of TcmJ in polyketide biosynthesis, a [his$_{10}$]-*tcmJ* overexpression plasmid in *E. coli* was made. A 1.4 kbp *Kpn*I−*Bam*HI fragment from pWHM765 (*7*) containing *tcmJ* was ligated into pGEM-7Zf. A 900 bp *Kpn*I−*Nar*I fragment in front of *tcmJ* was removed and replaced with a 51 bp linker containing an *Nde*I restriction site and sequence modifications that were more compatible with *E. coli* codon usage. The 540 bp *Nde*I−*Bam*HI fragment was inserted into pET16b (Novagen) to form plasmid pWHM1005. Transformation of the plasmid into *E. coli*, cell growth, induction of protein expression, and crude protein extraction were done as described above for the *fabD* plasmid. A 5 μL portion of the crude protein

Table 1: TCM PKS Activity in Reconstitution Assays

entry	proteins	rel yield of TCM F2 (%)[a]
1	TcmJKLMN	100[b]
2	[his$_6$]-TcmKL+TcmJΔ*K*LMN	100
3	[his$_6$]-TcmKL+TcmJK*Δ*LMN	100
4	TcmJΔ*K*LMN+TcmJK*Δ*LMN	−[c]
5	[his$_6$]-TcmKL+TcmM+TcmN+MAT	25[d]
6	[his$_6$]-TcmKL+TcmM+TcmN	−
7	TcmM+TcmN+MAT	−
8	[his$_6$]-TcmKL+TcmM+MAT	−[c]
9	[his$_6$]-TcmKL+TcmN+MAT	−
10	5+TcmJ	100
11	5+oxaloacetate+citrate synthase	25
12	5+acetyl CoA	25

[a] The actual yields were determined from the amount of radioactivity associated with the TCM F2 peak in the HPLC chromatogram (cf. Figure 3A and Figure 3C). Figure 3B represents the control for these experiments. [b] The same time and amounts of total protein and [2-^{14}C]malonyl-CoA were used in each assay for entries 1−4, but the amounts of TcmJ, TcmKL, TcmM, TcmN, and FabD MAT are unknown. Therefore, the relative yields for these entries may not be comparable with those for entries 5−12. [c] A small amount of TCM F2 was produced. [d] The same time and amounts of each protein and [2-^{14}C]malonyl-CoA were used in each assay for entries 5−12.

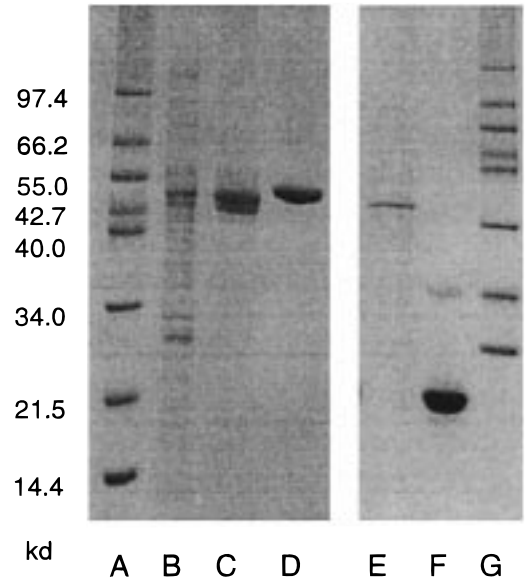

FIGURE 1: SDS−PAGE of TCM PKS proteins. Lanes A and G, molecular mass markers; lane B, TcmKL after DEAE-agarose chromatography; lane C, purified [his$_6$]-TcmKL; lane D, TcmN; lane E, MAT; lane F, TcmM.

extract (3 mg/mL) was used in the reconstitution reactions. Reaction initiation, termination, ethyl acetate extraction, and HPLC analysis were done as described above. To examine the necessity for acetyl-CoA in the production of TCM F2, oxaloacetic acid (final concentration, 100 μM to 1 mM) and citrate synthase (Sigma, 0.1−1 unit) were added to the malonyl-CoA, and this mixture was added to the reconstitution reaction. A coupled malate dehydrogenase and citrate synthase assay was used to measure the amount of acetyl-CoA available to the TCM PKS reactions. The assay solution consisted of 6.7 mM malate, 1.7 mM NAD, 56 units of malate dehydrogenase, and 0.1 unit of citrate synthase in 20 mM sodium phosphate buffer (pH 7.2). The assays were initiated by addition of acetyl-CoA or malonyl-CoA into the solution in cuvettes to measure the absorbance change at 340 nm.

RESULTS AND DISCUSSION

Properties of TcmK and TcmL Complex. To purify TcmK and TcmL, we first developed an assay to identify the activity of the enzymes on the basis of their ability to restore TCM F2 production in cell-free extracts lacking either protein but containing all of the other components of the TCM PKS (*11*). Since genetic studies had shown that at least the TcmK, TcmL, TcmM, and TcmN proteins were required, plasmids for overexpression of the *tcmJΔKLMN* or *tcmJKΔLMN* genes were constructed and introduced into *S. lividans* by transformation and cell-free extracts prepared as described (*11*). When crude protein extracts made from recombinant *E. coli* or *Streptomyces* strains overexpressing either of the native *tcmK* or *tcmL* genes were added to the latter extracts, TCM F2 biosynthesis was not restored. Furthermore, crude protein extracts from the strain with the *tcmJΔKLMN* genes did not complement the protein extract made from the *tcmJKΔLMN* strain to make TCM F2 in vitro (Table 1). Since these results suggested that we could not study TcmK and TcmL separately, we overexpressed the *tcmKL* genes together in *S. lividans* to obtain the KS subunits. The crude protein preparation made from the latter strain was able to complement both deletion mutants to make TCM F2 in vitro, as did the preparation containing [his$_6$]-TcmK plus TcmL (Table 1).

Successful development of this assay allowed purification of the TcmK and TcmL protein complex. After fractionation by 1.7 M ammonium sulfate precipitation, the TcmK/TcmL activity was found in the supernatant. Following hydrophobic interaction chromatography, the activity was recovered in the fractions with a low concentration of ammonium sulfate (<0.1 M). The TcmK/TcmL activity was further fractionated using gel filtration chromatography on Sephacryl S-200 and anion exchange chromoatography on DEAE-Bio Gel A agarose. After the latter step, TcmK and TcmL were the major bands observed upon SDS−PAGE analysis (Figure 1, lane B), suggesting that the KS subunits copurified. About 4 mg of protein was obtained at this step from a 1.2 L culture. To expedite further purification, [his$_6$]-TcmK/TcmL was produced and purified by nickel affinity chromatography. About 250 μg of [his$_6$]-TcmKL could be obtained from a 1.2 L culture. The nearly pure TcmKL and fully pure [his$_6$]-TcmKL complex (Figure 1, lane C) complemented protein extracts made from both *tcmJΔKLMN* and *tcmJKΔLMN* deletion mutant strains to make TCM F2 in vitro (Table 1).

The fact that TcmK and TcmL were copurified by the above procedure suggested the two proteins were associated in a complex. Sephacryl S-200 gel filtration chromatography was used to determine the molecular mass of the complex (Figure 2). The apparent molecular mass was 90 kd, suggesting an $\alpha\beta$ dimer containing 1 equiv each of TcmK (calculated molecular mass 45 kd) and TcmL (calculated mass 42 kd) in the complex. When purified [his$_6$]-TcmKL was analyzed by SDS−PAGE, equal amounts of TcmK and TcmL were seen, consistent with a 1:1 ratio of TcmK to TcmL in the complex (Figure 1, lane C). It is not known if the difference in the number of subunits in the KS complex between the ACT and TCM PKS has any particular significance; e.g., could it be related to the difference in the size of the polyketide framework made (octaketide vs

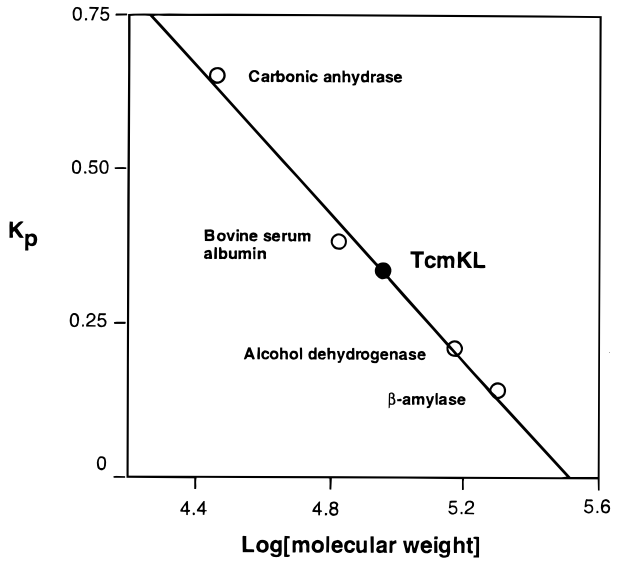

FIGURE 2: Estimation of the molecular weight of the TcmKL complex by gel filtration chromatography. $K_p = (V_e - V_o)/(V_t - V_o)$. V_o and V_t were determined using blue dextran and tyrosine, respectively.

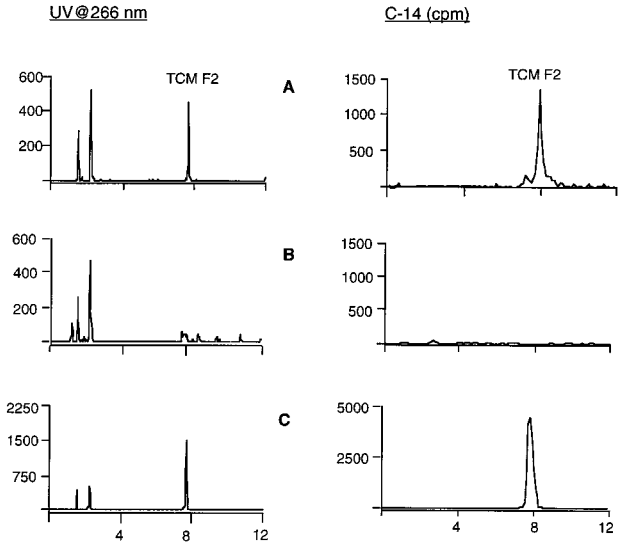

FIGURE 3: HPLC analysis with UV absorbance and ^{14}C radioactivity detection of TCM F2 production from malonyl-CoA in the TCM PKS reconstitution assay. (A) TcmKL + TcmM + TcmN + MAT; (B) TcmKL + TcmM + TcmN; (C) TcmKL + TcmM + TcmN + MAT + TcmJ. The *y* axes scales are in arbitrary units.

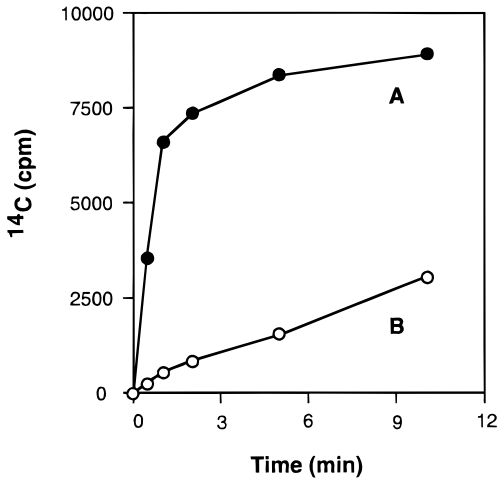

FIGURE 4: Acylation of TcmM with [2-^{14}C]malonyl-CoA. Reactions consisted of 2 μM malonyl-CoA (10 000 cpm), 2 mM DTT, and 5 μM TcmM in 20 mM phosphate buffer (pH 7.2). (A) Addition of 0.5 μM MAT; (B) without MAT.

decaketide) or the presence of the ActIII ketoreductase in the ACT PKS [ActIII is not absolutely required (*13*, *14*) but could be important for optimal activity]?

Reconstitution of TCM F2 Production. Purification of the TcmKL complex made the in vitro reconstitution of TCM PKS activity possible. Although we had established previously that a protein extract from a TCM PKS-minus strain overexpressing the *tcmKLMN* genes made TCM F2 in vitro (*11*), the absence of a MAT gene in the TCM gene cluster had led us to suggest that the *S. glaucescens* FabD MAT, located elsewhere in the chromosome, might catalyze the transfer of malonate from malonyl-CoA to TcmM as part of the TCM PKS mechanism (*11*, *18*). This possible link between secondary (PKS) and primary (fatty acid) metabolism was also noted for *S. coelicolor* (*15*). When the four TCM proteins and the MAT were added to the reaction solution along with malonyl-CoA, TCM F2 was produced (Figure 3A and Table 1). Reactions without the TcmKL complex, TcmM, TcmN, or MAT (Figure 3B and Table 1) resulted either in no TCM F2 production or only traces of it. Purified TcmM could complement the crude protein extract from strains containing only the TcmJ, TcmK, TcmL, and TcmN proteins (*11*), and purified TcmN could increase the amount of TCM F2 in vitro (*12*), indicating the participation of TcmM and TcmN in the production of TCM F2. Consequently, the results from our previous and present work prove that the TcmKL KS $\alpha\beta$ complex, TcmM ACP, TcmN cyclase, and FabD MAT proteins constitute the minimal requirements for a functional TCM PKS in vitro.

MAT Is Required for Acylation of the TcmM ACP. From the in vitro reconstitution experiments described above, the *fabD* MAT clearly is required for the reconstitution of TCM PKS activity (Figure 3B). However, self-catalytic acylation of several PKS ACPs with malonyl-CoA in the absence of an MAT has been reported recently (*16*). We also observed that TcmM underwent self-acylation when incubated with [2-^{14}C]malonyl-CoA (Figure 4), but the rate of self-acylation was much slower than the reaction catalyzed by the MAT. The initial rate of TcmM acylation in the reaction with the *fabD* MAT was 20 times faster than the reactions without this MAT. For example, when 5 μM TcmM was incubated with 2 μM [2-^{14}C]malonyl-CoA, 35% of the substrate was incorporated into TcmM in 10 min in the reactions without MAT, while 80% of the [2-^{14}C]malonyl-CoA was incorporated in 1 min in the reactions with 0.5 μM MAT (Figure 4). Self-malonylation of TcmM could not account for the amount of transacylation needed to make the quantity of TCM F2 we observed by HPLC. In the PKS reconstitution assay, 150 μM malonyl-CoA was normally in the reaction solution, and about 50% of the radioactivity was incorporated into TCM F2 in 1 h. Based on these data, it can be calculated that self-acylation only could account for $<10\%$ of the malonyl-CoA incorporated into TCM F2.

The *fabD* MAT gene is found as part of putative FAS gene clusters in both *S. glaucescens* (*18*) and *S. coelicolor* (*15*). These genes are remote from the TCM and ACT gene clusters, but MAT homologues are present in some other clusters of antibiotic biosynthesis genes made by type II

PKSs (*23*−*25*) and in the case of daunorubicin are known to be essential for its production (*26*). The FabD MAT enzyme was studied in the TCM PKS system because of its ability to catalyze the transacylation of TcmM. Carreras and Khosla (*14*) showed that the *S. coelicolor fabD* MAT was isolated as an essential component of ACT PKS activity and suggested that this MAT is one possible regulatory point for the control of primary and secondary metabolic flux. They further demonstrated that the MAT could covalently bind malonyl-CoA before transfer of the malonate to the ACT ACP. Although we could not observe any specific interaction between the *S. glaucescens fabD* MAT and malonyl-CoA (W.B. and C.R.H., unpublished data), the requirement for this MAT in the TCM PKS reconstitution assay further demonstrates the necessity of this primary biosynthesis enzyme in secondary metabolism.

Acetyl-CoA Is Not Required for the Production of TCM F2. Acetyl-CoA, the presumed starter unit for the TCM decaketide intermediate, was not a required substrate for the TCM PKS to produce TCM F2 in the reconstitution assay. When [1-^{14}C]acetyl-CoA was incubated with the reaction mixture 30 min before the addition of malonyl-CoA, a low incorporation of radioactivity into TCM F2 was detected (data not shown), which may be due to dilution of the acetyl-CoA pool by the decarboxylation of malonyl-CoA. When [1-^{14}C]acetyl-CoA and malonyl-CoA were added to the reaction at the same time, no radioactive TCM F2 was detected, and inclusion of acetyl-CoA in the reaction did not produce a different amount of TCM F2 (Table 1). Nonetheless, these results do not exclude the participation of acetyl-CoA in the reaction since it still could arise from the decarboxylation of malonyl-CoA to serve as the starter unit. In the tricarboxylic acid cycle, acetyl-CoA and oxaloacetic acid form citric acid by a Claisen condensation, catalyzed by citrate synthase. Since the reaction highly favors citrate formation thermodynamically (*27*), to test whether acetyl-CoA was necessary for the production of TCM F2 in our reconstitution assay, we mixed oxaloacetic acid and citrate synthase with malonyl-CoA and then added this mixture to the TCM PKS reconstitution assay solution. This did not affect the amount of TCM F2 produced (Table 1). As a control, we tested the activity of citrate synthase with the amount of acetyl-CoA in the malonyl-CoA sample due to decarboxylation. By coupling citrate formation with a spectrophotometric assay for malate dehydrogenase activity in the presence of citrate synthase (*28*), we could detect acetyl-CoA at a concentration as low as 2 μM in the phosphate buffer (pH 7.5) used. This allowed us to determine that the amount of acetyl-CoA in malonyl-CoA used in our reconstitution assay was about 1.5%. Spontaneous decarboxylation of malonyl-CoA was not detectable at room temperature in an assay that contained as much as 1 mM malonyl-CoA. In the TCM PKS reconstitution reactions, there was about 2 μM acetyl-CoA present in the 150 μM malonyl-CoA used in the assay, which could not account for the amount of TCM F2 produced (8−10 μM). We also tried to examine if the TcmKL complex could bind [^{14}C]-acetyl-CoA in the presence of the MAT in an assay performed in the same way as the holo-TcmM assay. No specific binding was observed between acetyl-CoA and TcmKL with or without the MAT over a period of 30 s to 30 min. These results are consistent with our earlier report that mutation of the Ser-351 residue to Ala in a putative acyltransferase site in the C-terminus of TcmK (*17*) did not affect the biosynthesis of Tcm F2 in vivo (*29*). This observation has been confirmed in vitro (W.B. and C.R.H., unpublished data). Since acetyl-CoA cannot be used directly by the TCM PKS, it is likely that the acetate starter unit arises by decarboxylation of malonyl-TcmM. Hints in favor of this idea have been reported earlier (*11*, *13*), and it is supported by the fact that acetyl-ACP, and not acetyl-CoA, is a kinetically competent intermediate of the ACT PKS in vitro (*30*). Precedents for the origin of propionate starter units from the decarboxylation of 2-methylmalonyl-CoA have been described for modular type I PKSs (*3*, *4*). In contrast, the type II PKSs for the aromatic polyketides that use propionate (daunorubicin) or malonamide (oxytetracycline) as a starter unit may require a dedicated MAT (*23*−*26*), or even a KS different from the TcmKL homologues (*23*, *24*, *26*), to initiate polyketide synthesis since neither of these starter units can be produced directly from malonyl-ACP.

TcmJ Increased the Production of TCM F2 but Could Not Replace the TcmN Cyclase. The biochemical function of TcmJ is not clear from our previous studies. Although its gene lies immediately upstream of the *tcmKLMN* genes, a strain with an in-frame deletion in *tcmJ* still made TcmC (*31*). Addition of *tcmJ* to the *tcmKLMN* gene cassette expressed in *Streptomyces* sp. increased the production of TCM F2 in vivo (*31*, *32*) and in vitro (*11*). Because of this apparent enhancement of PKS activity, TcmJ has been presumed to have a possible role in the cyclization of the polyketide backbone. In our reconstitution assay, addition of partially purified [his$_6$]-TcmJ to the reaction greatly increased the production of TCM F2 (Figure 3C, Table 1), but when the assay contained TcmJ instead of TcmN, no more TCM F2 was produced than in the reactions without TcmJ or TcmN (W.B. and C.R.H. unpublished data). Consequently, it seems that TcmJ is an architectural protein that enhances TCM PKS activity without playing a catalytic role, and not just evolutionary detritus.

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