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Deciphering the mechanism for the assembly of aromatic polyketides by a bacterial polyketide synthase

(antibiotic/secondary metabolite/*Streptomyces glaucescens*/tetracenomycins)

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ABSTRACT **Aromatic polyketides are assembled by a type II (iterative) polyketide synthase (PKS) in bacteria. Understanding the enzymology of such enzymes should provide the information needed for the synthesis of novel polyketides through the genetic engineering of PKSs. Using a previously described cell-free system [B.S. & C.R.H. (1993) *Science* 262, 1535–1540], we studied a PKS enzyme whose substrate is not directly available and purified the TcmN polyketide cyclase from *Streptomyces glaucescens*. TcmN is a bifunctional protein that catalyzes the regiospecific cyclization of the Tcm PKS-bound linear decaketide to Tcm F2 and the *O*-methylation of Tcm D3 to Tcm B3. In the absence of TcmN, the decaketide formed by the minimal PKS consisting of the TcmJKLM proteins undergoes spontaneous cyclization to form some Tcm F2 as well as SEK15 and many other aberrant shunt products. Addition of purified TcmN to a mixture of the other Tcm PKS components both restores and enhances Tcm F2 production. Interestingly, Tcm F2 but none of the aberrant products was bound tightly to the PKS. The results described support the notion that the polyketide cyclase, not the minimal PKS, dictates the regiospecificity for the cyclization of the linear polyketide intermediate. Furthermore, because the addition of TcmN to the TcmJKLM proteins results in a significant increase of the total yield of decaketide, interactions among the individual components of the Tcm PKS complex must give rise to the optimal PKS activity.**

Polyketide metabolites are one of the largest groups of natural products, many of which are clinically valuable antibiotics or chemotherapeutic agents, or exhibit other pharmacological activities (1, 2). Despite their apparent structural diversity, they share a common mechanism of biosynthesis. The carbon backbone of a polyketide results from sequential condensation of short fatty acids, such as acetate, propionate, or butyrate, in a manner resembling fatty acid biosynthesis but is catalyzed by polyketide synthases (PKSs). A strong sequence and mechanistic similarity among many of the PKSs has led to two paradigms for explaining polyketide biochemistry: type I PKSs, multifunctional proteins that harbor a distinct active site for every enzyme-catalyzed step, and type II PKSs, multienzyme complexes that carry a single set of iteratively used activities and consist of several largely monofunctional proteins for the synthesis of complex largely reduced polyketides, like macrolide antibiotics, or aromatic polyketides, like tetracyclines (3, 4, 5). Studies of polyketide biosynthesis provide an excellent model for elucidating the structure–function relationship of complex multienzyme systems, and engineered PKSs have considerable potential for synthesizing novel polyketides (6–9).

So far, the examination of type II PKS genes *in vivo* has revealed limited information about how type II PKSs control product structure, in part because the active sites for the RCOSEnz and $CH_2(CO_2H)COSEnz$ condensation and oligoketide cyclization reactions are used more than once and, therefore, must recognize different substrates in each bond-forming event, as exemplified by the tetracenomycin (Tcm) PKS from *Streptomyces glaucescens* (see Fig. 1) (10–12). We have previously described a cell-free system to facilitate the purification and reconstitution of individual components of type II PKSs (11), using the Tcm PKS as a model. That work showed that the Tcm PKS consists of the TcmJKLMN proteins (10, 12–14), although components of a *S. glaucescens* fatty acid synthase may also be required (11, 15). We now report the purification of the TcmN polyketide cyclase and its interaction with the other Tcm PKS proteins (i.e., TcmJKLM), which reveals important mechanistic details about how a type II PKS assembles aromatic polyketides.

MATERIALS AND METHODS

Expression of *tcmN* in *Streptomyces lividans* and Purification of TcmN. A 0.5-kb fragment containing the *ermE** promoter was subcloned from pWHM862 (16) into the *Eco*RI/*Sst*I sites of pGEM3zf (Promega) to give pWHM79. The *tcmN* gene was then moved as a 1.8-kb *Bgl*II/*Sph*I fragment from pWHM862 into the *Bam*HI/*Sph*I sites of pWHM79 to give pWHM80, from which a 2.3-kb *Bam*HI/*Eco*RI fragment containing *ermE*::tcmN* was moved into the similar sites of pWHM862 to yield pWHM74. Transformation of pWHM74 into *S. lividans* 1326, preparation of cell-free extract from *S. lividans* 1326 (pWHM74), and SDS/PAGE analysis were performed as described (17). The cell-free extract was fractionated by addition of solid $(NH_4)_2SO_4$. The pellet resulting from 31% to 46% saturation was collected, dissolved in a minimum amount of 25 mM Tris·HCl buffer (pH 8.0)/1 mM DTT/150 mM NaCl, and resolved on a Sephacryl S-200 HR column (2.6 × 60 cm) eluted with the same buffer. The TcmN-containing fractions after the gel filtration chromatography, assayed as described below, were loaded onto a Mono Q HR 10/10 (Pharmacia) column in 25 mM Tris·HCl buffer (pH 8.0)/1 mM DTT and eluted at a flow rate of 2 ml/min with a linear 80-ml gradient from 0.1 to 0.5 M NaCl in the same buffer. Fractions containing TcmN from the anion exchange chromatography were brought to 1.0 M $(NH_4)_2SO_4$, applied to an Alkyl Superose HR 5/5 (Pharmacia) column, and eluted at a flow rate of 0.5 ml/min with a linear 15-ml gradient from 1.2 to 0.48 M $(NH_4)_2SO_4$ in 50 mM sodium phosphate buffer (pH 7.2)/1.0 mM DTT to yield pure TcmN.

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Abbreviations: PKS, polyketide synthase; Tcm, tetracenomycin.

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The molecular weight of the native TcmN was determined by gel filtration chromatography on a Sepharose 6 HR 10/30 (Pharmacia) column. The column was eluted with a flow rate of 0.5 ml/min in 25 mM Tris·HCl buffer (pH 8.0)/150 mM NaCl/1 mM DTT and calibrated with blue dextrin (2×10^6), alcohol dehydrogenase (M_r 150,000), BSA (M_r 66,000), and carbonic anhydrase (M_r 29,000); (Sigma).

Expression of *tcmN* in *Escherichia coli*. Amplification of the *tcmN* gene by PCR was performed as described (17). The sequences of the primers were as follows (restriction enzyme sites are underlined and modified bases are shown in bold): 5′-GGAATTCCATGGCTGCTCGTACCGACAACTCC-ATCGTCGTCAACGCGCCGTTC-3′ and 3′-ACCTGGTG-CGACCTCACGGCGGGGCAGATCCTAGGTCG-5′. The amplified 1.5-kb *tcmN* fragment was digested with *Nco*I and *Bam*HI and cloned into the similar sites of pET14b (Novagen) to yield pWHM78. Transformation of pWHM78 into *E. coli* BL21(DE3) and preparation of cell-free extract from *E. coli* BL21(DE3)(pWHM78) were carried out on the pET system according to the manufacturer's instructions (Novagen). TcmN was then purified as above.

Assay for TcmN *O*-Methyltransferase Activity. To assay the methyl transferase activity of TcmN, a 500-μl solution with 5% dimethyl sulfoxide consisting of 100 μM Tcm D3 (18), 250 μM *S*-adenosylmethionine, and 2.6 μg of TcmN in 50 mM Tris·HCl buffer (pH 8.0) was incubated at 25°C. After 10 min of preincubation, the assays were initiated by addition of Tcm D3, incubated at 25°C, and terminated by addition of 50 μl of 20% trichloroacetic acid. They were then saturated with NaCl and extracted with EtOAc (2×500 μl), and the EtOAc extracts were concentrated *in vacuo*. The latter residues were dissolved in CH_3OH and analyzed by HPLC under the conditions described previously (17). Tcm D3 and Tcm B3 (19) have retention times of 7.4 min and 8.8 min, respectively.

Assay for Tcm Polyketide Cyclase Activity. Cell-free preparation of *S. glaucescens* WMH1077(pWHM731) and enzyme assay of the Tcm PKS and the TcmN cyclase activities were performed as described (ref. 11; see also the footnote of Fig. 3).

RESULTS AND DISCUSSION

Purification and Characterization of TcmN on the Basis of Its *O*-Methyltransferase Activity. We took advantage of the bifunctional nature of TcmN (12) and purified it on the basis of its *O*-methyltransferase activity (Fig. 1*A*). The *tcmN* gene was overexpressed in both *S. lividans* and *E. coli* to facilitate its purification. In *S. lividans*, the expression of *tcmN* was under the control of the strong, constitutive *ermE** promoter in pWHM74 (see *Materials and Methods*). Analysis of a cell-free sample made from *S. lividans* 1326 transformed with pWHM74 by SDS/PAGE showed a distinctive band that migrated with a M_r of 56,000, indicating that the *tcmN* gene had been overexpressed in the heterologous host in the soluble form (Fig. 2*A*). Purification of the TcmN protein to homogeneity was accomplished by sequential gel filtration, anion exchange, and hydrophobic interaction chromatography as described in *Materials and Methods* and summarized in Table 1. Amino acid analysis of the purified protein yielded a N terminus of AARTDNSIVVNAPEELVXDVTN, confirming the translational start site assigned previously to *tcmN* based on genetic analysis (12). We then used PCR to create a unique *Nco*I site at the ATG start site and a *Bam*HI site after the TAG stop site to facilitate the construction of pWHM78 for *tcmN* expression in *E. coli* as described in *Materials and Methods*. Codons 2–5 of the *tcmN* gene were also modified for the optimal expression in *E. coli* (20). SDS/PAGE analysis of samples from *E. coli* BL21(DE3) transformed with pWHM78 showed that the *tcmN* gene was efficiently expressed and that approximately 90% of the TcmN protein was produced in the soluble form (Fig. 2*B*), from which it was purified to homogeneity by the procedures used for TcmN from *S. lividans* (Table 1).

Since the most likely substrate for the TcmN polyketide cyclase would be the PKS-bound linear decaketide (Fig. 1*A*), which cannot be prepared chemically because of its inherent reactivity, we purified TcmN by following the Tcm D3 *O*-methyltransferase activity. In agreement with our earlier prediction (12), the purified TcmN protein catalyzed the specific *O*-methylation of Tcm D3 to yield Tcm B3 (Fig. 1*A*), using *S*-adenosylmethionine as the methyl donor, and showed optimal activity around pH 8.0. TcmN produced in either *S. lividans* or *E. coli* was identical, as evidenced by a similar specific activity of 26.0 or 27.8 nmol·min^{-1}·mg^{-1}, respectively, in the Tcm D3 *O*-methyltransferase assay (Table 1).

TcmN Polyketide Cyclase Activity. Genetic studies *in vivo* fall short in identifying the actual substrate for a polyketide cyclase like TcmN because the corresponding mutants in the cyclase genes have resulted in the formation of aberrantly

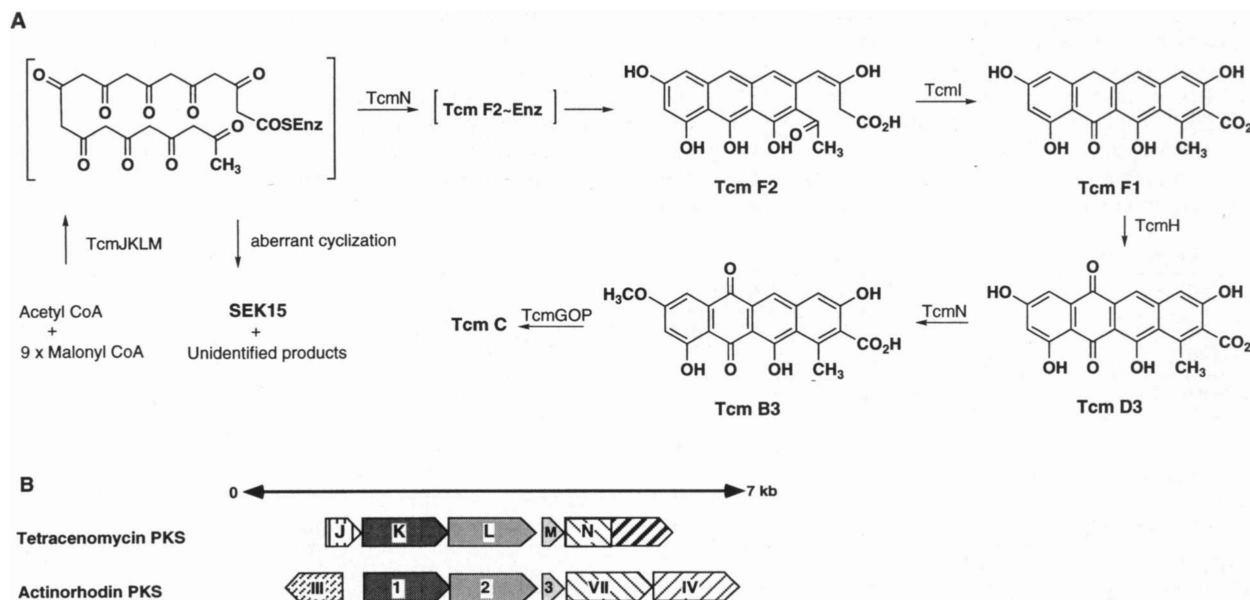

FIG. 1. (*A*) Biosynthetic steps of Tcm C in *S. glaucescens*. (*B*) Genes for the Tcm PKS in *S. glaucescens* and the actinorhodin PKS in *Streptomyces coelicolor* (8).

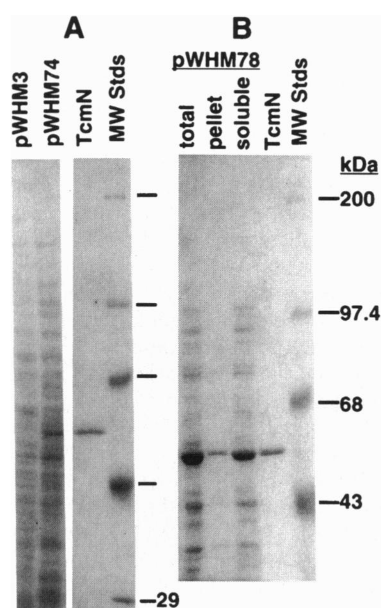

FIG. 2. Overexpression of the *tcmN* gene in *S. lividans* and *E. coli* analyzed by SDS/PAGE (7.5% polyacrylamide gels). (*A*) Cell extracts from *S. lividans* containing pWHM3 (vector) or pWHM74. Purified TcmN and molecular weight (MW) standards (myosin H-chain, 200,000; phosphorylase *b*, 97,400; BSA, 68,000; ovalbumin, 43,000; carbonic anhydrase, 29,000). (*B*) Cell extracts from *E. coli* BL21(DE3) (pWHM78) representing total proteins, protein pellet, and supernatant proteins after centrifugation at 27,500 × *g*; purified TcmN and MW standards.

cyclized shunt products, although the existence of the enzyme-bound linear polyketide (Fig. 1*A*) has been assumed. We reported earlier that the *S. glaucescens* WMH1077(pWHM731) transformant carrying the *tcmJKLM* genes synthesized decaketide metabolites *in vivo* (9); its cell-free preparation catalyzed the synthesis of apparent decaketide metabolites from acetyl- and malonyl-CoA *in vitro* (11), implying that the TcmJKLM proteins are competent to synthesize the full-length decaketide in the *S. glaucescens* WMH1077 background. Based on these results, the nascent linear decaketide (Fig. 1*A*) is likely to be the direct substrate for TcmN, which in the absence of any cyclase could slowly undergo aberrant cyclizations as evidenced by the observation of a mixture of cyclized compounds among which only Tcm F2 (18) and SEK15 (21) have been identified (9). To test this hypothesis, we supplemented the cell-free preparation of *S. glaucescens* WMH1077 (pWHM731) with purified TcmN with the anticipation that TcmN would subvert all aberrant reactions by regiospecifically cyclizing the linear decaketide into Tcm F2. As shown in Fig. 3*D*, Tcm F2 was by far the major product synthesized, and all other patterns of cyclization, including the one leading to the formation of SEK15 (Fig. 3*C*), were diminished below the limits of detection. These data support the existence of the putative decaketide and demonstrate that a cyclase like TcmN is largely responsible for the regiospecific cyclization of the linear polyketide intermediate.

We wished to determine whether both the linear polyketide intermediate and Tcm F2 are enzyme-bound. If the former were true, as heretofore predicted (3–5), the decaketide, or more likely its aberrantly cyclized products, should be isolated along with the PKS enzymes in the absence of the TcmN cyclase. A cell-free preparation of *S. glaucescens* WMH1077(pWHM731) was incubated with acetyl- and malonyl-CoA for 20 min, during which a steady state of enzyme-bound intermediate was presumably established. Substrates and products free from the PKS complex were removed by passing the incubation mixture through a Sephadex G-25 column (Pharmacia), and the resulting enzyme solution was saturated with NaH_2PO_4 and denatured by extraction with EtOAc to isolate any noncovalently enzyme-bound products. Surprisingly, Tcm F2 was the only enzyme-bound product observed (Fig. 3*E*) despite the fact that a complex mixture consisting of Tcm F2, SEK15, and other unidentified products was synthesized in the assay reaction and recovered by solvent extraction (Fig. 3*C*). Since SEK15 and the products other than Tcm F2 did not appear with the eluted protein (Fig. 3 *C* vs. *E*), Tcm F2 appears to adhere to the Tcm PKS by a tight noncovalent interaction. Covalent attachment by a thioester bond to the 4′-phosphopantetheinyl-SH group of the TcmM acyl carrier protein (13) is not excluded by the data but is less likely because this bond should have been stable to the isolation conditions (22). Regardless, the normal product Tcm F2 clearly is bound much tighter than the aberrant products. This result could mean that the PKS complex has a specific binding pocket fitting only the normal product, Tcm F2, and forcing the abnormally cyclized ones like SEK15 off the enzyme complex. This binding pocket might also fold the decaketide intermediate into the shape leading to the linearly fused ring system of Tcm F2.

When this experiment was repeated under the identical conditions with the addition of purified TcmN, Tcm F2 was the sole enzyme-bound product again, but the amount of Tcm F2 trapped was more than 10-fold higher than that from TcmJKLM alone (Fig. 3 *F* vs. *E*). From these results, we conclude that the linear decaketide intermediate, the actual substrate for the TcmN cyclase, is enzyme-bound, most likely as an acylthioester to TcmM. In the absence of a cyclase, it cannot efficiently undergo regiospecific cyclization to Tcm F2 and, as a result, falls off the enzyme complex either before or during the aberrant cyclization, as evident from the fact that no other cyclized product apart from Tcm F2 appeared to be enzyme-bound.

Table 1. Purification of TcmN

	10 g of *S. lividans* 1326 (pWHM74) cells*					3.5 g of *E. coli* BL21 (DE3) (pWHM78) cell*				
Step	Protein†, mg	Units‡	Spec. act.§	Yield, %	Purification, *x*-fold	Protein†, mg	Units‡	Spec. act.§	Yield, %	Purification, *x*-fold
Cell-free extract	480	251	0.523	100	1.00	308	2.72	8.83	100	1.0
$(NH_4)_2SO_4$ fractionation	244	209	0.857	83.3	1.64	112	1.70	15.2	62.5	1.72
Sephacry S-200	36.0	193	5.36	76.9	10.2	64.0	1.65	25.8	60.7	2.92
Mono Q HR 10/10	8.64	103	11.9	41.0	22.8	40.5	1.11	27.4	40.8	3.10
Alkyl Superose HR 5/5	3.07	80.0	26.0	31.9	49.7	25.3	0.703	27.8	25.8	3.15

*Wet weight.

†Total proteins were determined by the method of Bradford with BSA as standard.

‡One unit of TcmN activity as an *O*-methyltransferase is defined as the formation of 1 nmol of Tcm B3 per min or the consumption of 1 nmol of Tcm D3 per min.

§Specific activity is defined as 1 unit/mg of TcmN.

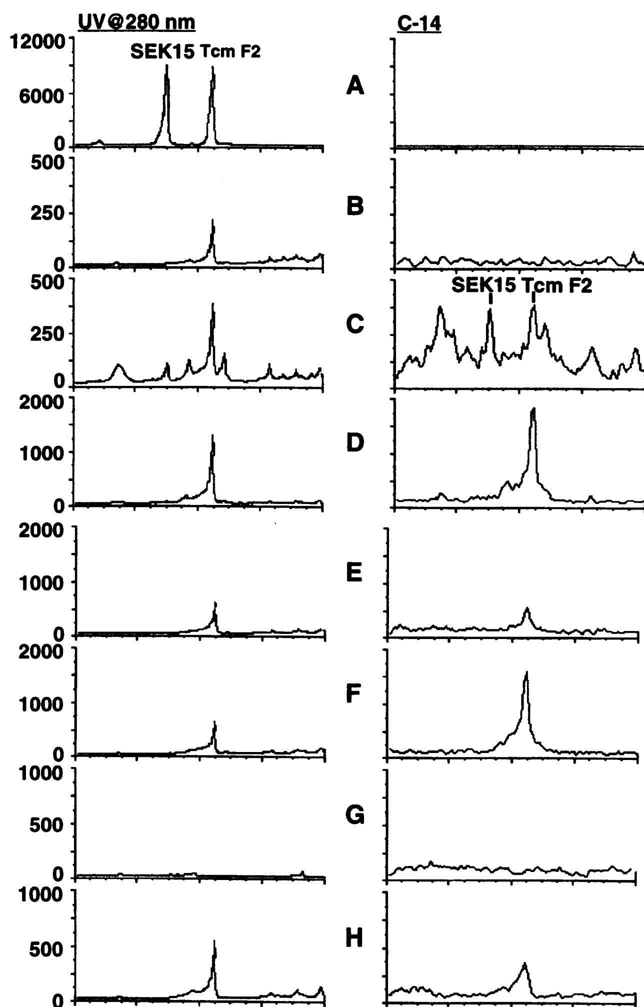

FIG. 3. HPLC analysis with UV absorbance and ^{14}C-radioactive detection of the *in vitro* synthesis of Tcm F2, SEK15, and other decaketides from acetyl-CoA and malonyl-CoA. The complete assay solution (500 μl) consisted of 50 μM acetyl-CoA, 150 μM [2-^{14}C]malonyl-CoA (with the indicated amount of radioactivity), 2 mM DTT, the indicated amount of total protein from *S. glaucescens* WMH1077-(pWHM731) containing the Tcm PKS, and purified TcmN in 0.1 M sodium phosphate buffer (pH 7.5). After 30 min of preincubation, the assays were initiated by addition of malonyl-CoA. (*B–D*) The assay solutions were incubated at 30°C for 90 min and terminated by addition of solid NaH_2PO_4 to saturation and extraction with EtOAc (2 × 400 μl). (*E* and *F*) The assay solutions were incubated at 30°C for 20 min followed by a passage through a Sephadex G-25 column; the resulting solutions were worked up as above. (*G* and *H*) The assay solutions were incubated at 30°C for 20 min and passed through a Sephadex G-25 column, and 100 μg of TcmN was added, followed by incubation at 30°C for an additional 20 min. The assay solution was then added to a Centricon 10 device to separate the proteins from the solution; the retained proteins, dissolved in 500 μl of H_2O, and the solution were worked up as above. The EtOAc extracts were concentrated *in vacuo* to dryness, dissolved in 80 μl of CH_3OH, and analyzed by HPLC on a Waters Radi-Pak C_{18} column (Novapak, 4 μM, 8 × 100 mm). The column was developed with a linear gradient of $CH_3CN/H_2O/CH_3CO_2H$ from 10/90/0.1 to 35/65/0.1 in 15 min, under which Tcm F2 and SEK15 were eluted with retention times of 9.5 min and 12.3 min, respectively. (*A*) SEK15 and Tcm F2 standards; (*B*) 150 μg TcmJKLM proteins and no malonyl-CoA added; (*C*) 2 × 10^4 cpm malonyl-CoA and 150 μg of TcmJKLM proteins; (*D*) 2 × 10^4 cpm malonyl-CoA, 150 μg of TcmJKLM proteins, and 50 μg of TcmN; (*E*) 2 × 10^5 cpm malonyl-CoA and 0.9 mg of TcmJKLM proteins; (*F*) 2 × 10^5 cpm malonyl-CoA, 0.9 mg of TcmJKLM proteins, and 200 μg of TcmN; (*G*) 1 × 10^5 cpm malonyl-CoA and 0.6 mg of TcmJKLM proteins—material recovered from the solution; (*H*) 1 × 10^5 cpm malonyl-CoA and 0.6 mg of TcmJKLM proteins—material recovered from the proteins retained on filter.

Genetic analysis of type II PKS clusters has yet to reveal any protein motif indicative of thioesterase activity, which presumably could be responsible for the release of the decaketide or Tcm F2 from TcmM. [Although TcmK contains a esterase-like motif that might serve this function, replacement of the predicted active serine with an alanine did not reduce the amount of Tcm F2 or Tcm C made by the *tcmK* mutant compared with that formed by the wild-type strain (16).] Working on the assumption that the enzyme-bound Tcm F2 above was attached to TcmM as a thioester, we tested whether TcmN could function as such a thioesterase by incubating a sample of the enzyme-bound Tcm F2 in the presence of TcmN for 20 min. If TcmN has thioesterase activity, Tcm F2 should have been released from the enzyme into the assay solution. Separation of the solution from the proteins was accomplished by passage through a Centricon 10 device (Amicon). Analysis of both the filtrate and protein portions showed that no Tcm F2 was released into the assay solution (Fig. 3*G*) and that all the Tcm F2 was still bound to the enzyme (Fig. 3*H*). Release of this bound Tcm F2 by the method used to recover the EtOAc soluble materials is not surprising because the proteins would have been denatured, weakening the Tcm F2~enzyme bond (Fig. 1*A*).

We cannot explain how Tcm F2 is released *in vivo* to be acted upon by the TcmI cyclase that uses free Tcm F2 as substrate (ref. 23; Fig. 1*A*). Spontaneous release or release by a thioesterase activity of a fatty acid synthase are two possibilities. Among the known fungal type I PKSs that produce aromatic compounds, a thioesterase-like domain can be recognized in some (PKSst; ref. 24) but not all [6-MSAS, ref. 25; WA, ref. 26; and PKS1, ref. 27) of the deduced protein sequences, and its presence does not seem to be correlated with how the polyketide is made. Moreover, 6-methylsalicylic acid is also released from the fungal PKS *in vitro* without the apparent need for a thioesterase (28).

We also examined the idea that the interaction of TcmN with TcmJKLM is essential for the Tcm PKS to efficiently assemble the decaketide from acetyl- and malonyl-CoA. Based on *in vivo* results, Khosla, Hopwood, and coworkers (8) and we (9) have concluded that the "minimal PKS" (i.e., the TcmKLM proteins or their closely related proteins in other type II PKSs) is responsible for the assembly of a given length linear polyketide. Furthermore, we believe that the regiospecificity of the subsequent cyclizations is determined only by the polyketide cyclases. However, these data neither reveal how the linear polyketide is channeled from the minimal PKS to the cyclase nor prove that the synthase and cyclase activities function autonomously. The data presented above confirm the polyketide cyclase-directed regiospecific cyclization of an enzyme-bound polyketide yet raise the question of whether the cyclase also influences how the minimal PKS assembles the linear decaketide, a question that can be addressed only with purified proteins. It is clear by comparing Fig. 3 *C* with *D* and *E* with *F* that addition of TcmN to the TcmJKLM proteins not only alters the product from a mixture (Fig. 3*C*) to largely Tcm F2 (Fig. 3*D*), as expected from the cyclase activity, but also increases the total yield of the polyketide synthesized from acetyl- and malonyl-CoA 5- to 10-fold (the variation observed between experiments). Since TcmN does not appear to be able to speed up the synthesis by facilitating the release of Tcm F2 from the PKS enzyme, the increased production of Tcm F2 must reflect increased ability to assemble the linear decaketide from acetyl- and malonyl-CoA. (This prediction cannot be verified by measuring enzyme turnover rates because the amount of Tcm PKS in our cell-free preparation is not known nor easily determined.) Therefore, we propose that TcmN is an integral part of the Tcm PKS complex, although it does not directly catalyze assembly of the linear decaketide. Although the TcmJKLM proteins can form a complex to assemble the decaketide inefficiently and form some Tcm F2 spontaneously,

it is the interactions between the TcmN and TcmJKLM proteins that constitute an optimal Tcm PKS complex. This model is in agreement with our finding that both the linear decaketide and Tcm F2 appear to be enzyme-bound, implying the close proximity between TcmN and the minimal Tcm PKS proteins, and is consistent with the *in vivo* studies (9, 29) in which addition of cyclases to a minimal PKS construct caused an increase in the production of metabolites.

Sequence analysis of the *tcmN* gene (12) showed that only the N-terminal portion of the deduced amino acid sequence is homologous to other putative polyketide cyclase genes or their closely related aromatases (30), such as the ActVII protein from *S. coelicolor* (31). The latter type of proteins exhibits an internal "duplication" motif in which the sequences of the N- and C-terminal halves of the protein are similar (32). One hypothesis for this sequence duplication is that the two halves could fold internally to form the active conformation. For TcmN to achieve a similar conformation, two molecules of TcmN would be required, each one providing half of the homodimer. This prediction is supported by the size of TcmN determined by gel filtration chromatography (*Materials and Methods*), where it eluted with an apparent M_r of 103,000, indicating that TcmN is indeed a homodimer in solution.

The results presented here and previously (11) reinforce the idea that the optimal Tcm PKS is a complex consisting of the TcmJKLMN proteins. It is the integrity of this complex that maximizes the efficiency for the synthesis of aromatic polyketides from acetyl- and malonyl-CoA. The nascent linear polyketide intermediate, whose length is determined by the TcmKL proteins (9, 33), appears to be enzyme-bound and is cyclized regiospecifically by the TcmN component of the very same complex. Disrupting or weakening the interactions among these proteins could either completely destroy the PKS activity, as noticed early in the constructions of an actinorhodin/tetracenomycin hybrid PKS (9), using genes shown in Fig. 1*B*, or lead to an inefficient PKS complex such as the TcmJKLM proteins reported here. Early departure of the decaketide intermediate from the PKS complex results in aberrant cyclization (8, 9), and this could lead to an erroneous conclusion about the enzymatic properties of a given cyclase or aromatase in hybrid PKSs. These findings must be now taken into consideration in future attempts to produce novel polyketides through the rational engineering of type II PKSs.

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